CRYOPRESERVATION OF CELL CULTURES AND EXPLANTS OF GRASSES

By

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DEDICATED TO MY LATE BELOVED FATHER, S.P. GNANAPRAGASAM

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TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School
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Methods were developed for the successful cryopreservation of whole immature embryos, embryogenic callus and cell suspension cultures of several gramineous species. The application of a pregrowth treatment, the use of cryoprotectants and the cooling rate of the tissue were all essential to success, but different cultures showed different requirements that had to be optimized for each.

A combination of cryoprotectants was needed for the cryopreservation of cell suspensions, but using a single cryoprotectant was best for the cryopreservation of callus and immature embryos. A pregrowth period in a liquid culture medium supplemented with an osmoticum was essential for recovery of callus cultures. Slow cooling was suitable for all types of cultures. Rapid cooling by direct immersion in liquid nitrogen was always lethal. Washing was invariably detrimental, and for callus cultures it was essential to plate the tissues with the cryoprotectants.

Plants were regenerated from a cryopreserved embryogenic cell suspension and a callus culture of a sugarcane commercial hybrid and from immature embryos of wheat. All were grown to maturity in the greenhouse and found to be morphologically similar to control plants.

A *Panicum maximum* cell suspension was used to study ultrastructural changes during the process of cryopreservation using light and electron microscopy.

Growing the cells in high osmoticum caused a reduction in cell size and vacuolar volume. Dilation of the ER and mitochondria was also observed at different stages of the cryopreservation procedure. Extensive damage was observed in cells that were frozen slowly without any cryoprotection or frozen by direct immersion after treatment with cryoprotectants. Osmiophilic granules were abundant in lethally damaged cells.

CHAPTER 1

INTRODUCTION

Cell cultures have to be routinely maintained for extended periods of time by periodic subculture, a time consuming and labor intensive process which can result in loss of the cultures due to microbial contamination, equipment failure or human error. Long term maintenance by subculture can also result in mutations including changes in the structure and number of chromosomes (Heinz and Mee 1971; Sheridan 1975; Sunderland 1977; Bayliss 1980; D'Amato 1985), and the consequent loss of morphogenetic potential (Murashige and Nakano 1967; Torrey 1967; Nag and Johri 1969; Meyer-Teuter and Reinert 1973; Smith and Street 1974; Reinert *et al.* 1977).

Crop improvement by conventional selection and breeding depends on reliable germplasm storage of the source material and improved genotypes (Withers 1988). For species that produce orthodox seeds, germplasm storage is achieved by gene-banks mostly composed of seed stores held at reduced temperature and humidity. Maintaining them in storage without physiological deterioration is an important but difficult task (Stanwood 1985). This method is not useful for species with recalcitrant seeds (coffee, coconut, oil palm, cocoa, rubber, mango, walnut etc.), which lack a dormancy period and are therefore incapable of surviving low temperatures and dehydration, and also for vegetatively propagated crops (Withers

1986). Such materials are normally stored in field gene-banks which are expensive to maintain and susceptible to environmental and pathogenic risks (Withers 1988).

Isolated shoot-tips (meristems) are often used for clonal propagation and production of virus free plants (Kartha and Gamborg 1975; Quak 1977; Roca *et al.* 1982; Kartha 1985a; Sakai 1985). Such cultures, and plants derived from them, cannot be maintained in a disease free and genetically stable condition for extended periods of time.

In vitro cultures are being increasingly used for the synthesis of secondary products which are of pharmaceutical and nutritional importance (Zenk 1978; Kurz and Constabel 1979; Staba 1980; Deus and Zenk 1982; Fowler 1986; Constabel and Vasil 1988). Therefore, periodic reselection or even the establishment of new cultures becomes necessary. The tendency of such cultures to lose the capacity to synthesize secondary products (Dhoot and Henshaw 1977) can result in the loss of valuable cell lines.

Haploid plants are important in the induction of mutations, identification of useful recessive genes, production of isogenic lines, and biochemical/genetic studies (Han 1983). They can be obtained from in vitro culture of microspores/anthers. They are often highly unstable, and rapidly become aneuploid and polyploid.

An in vitro method which could store material in a stable state for indefinite periods of time, in addition to being applicable to all types of cultures, would be ideal for overcoming most of the problems mentioned above. Two methods have been used for the storage of plant cell and tissue cultures: (1) reduction in the rate of growth (slow growth), and (2) suspension of growth.

Slow growth can be achieved by a reduction in incubation temperature or available oxygen, or by the application of osmotic inhibitors or hormonal retardants (Bridgen and Staby 1981; Withers 1985a, 1986). The most widely used method of slow growth is the reduction in culture temperature (Bannier and Steponkus 1972;

Meyer-Teuter and Reinert 1973; Hiraoka and Kodama 1982, 1984; Jacques *et al.* 1982). Plant cultures are normally grown at temperatures of 20 to 25°C, and lowering the temperature by 10-15°C results in a marked decrease in the rate of growth (Withers 1985a).

The mineral oil overlay technique, which reduces growth rates by limiting the availability of oxygen, is commonly used for storage of microbial cultures, and can be used to a limited extent for higher plant cell cultures (Caplin 1959; Augereau *et al.* 1986). Subculture intervals can be extended from weeks to months by a decrease in the growth rate by at least a factor of four (Dougall 1980). Osmotic (mannitol) or hormonal (ABA) inhibitors can be added to the culture medium to retard growth, as an alternative or in combination with reduced growth temperatures (Withers 1985a). Slow growth can be used for short to medium term storage, but long term storage requires periodic renewal, at least every one to two years. Therefore the problems encountered in maintaining the cell cultures by repeated subculture are not eliminated by growth limitation, but rather extended over a longer period of time (Withers 1985a). In addition, there is increased risk of selecting new variant cell lines, due to the stress imposed by the condition of culture (Withers 1984a). Slow growth has been mostly successful for storage of shoot cultures (Mullin and Schlegel 1976; Lundergan and Janick 1979; Dale 1980). It has shown only limited success with callus cultures (Bannier and Steponkus 1972, 1976; Hiraoka and Kodama 1984), and cannot be applied to cell suspension cultures (Rose and Martin 1975).

For complete avoidance of subculturing, the cultures have to be stored under conditions which involve the suspension of all metabolism (Withers 1983). This can be achieved by either freeze-drying or cryopreservation. Freeze drying, which is extensively used for conservation of microorganisms (Grout *et al.* 1990), is not adaptable to higher plant tissue cultures (Withers 1983). Furthermore, mutation

events associated with freeze drying have been documented (Ashwood-Smith and Grant 1976; Tanaka *et al.* 1979).

Cryopreservation, which eliminates all requirements for periodic transfer or viability testing by storing material at liquid nitrogen temperatures (liquid at -196°C and vapor at -150°C), can in theory store material for indefinite periods of time. Unlike slow growth, it is applicable to all types of cultures from protoplasts to plantlets, but unorganized cultures generally fare better than organized material (Withers 1987). At liquid nitrogen temperatures the kinetic energy levels are too low to allow the necessary molecular motions for the normal cellular chemical reactions to occur (Grout *et al.* 1990). Changes such as formation of free radicals and macromolecular damage due to ionizing radiation can still occur at liquid nitrogen temperatures (Grout *et al.* 1990), but the risk of occurrence of genetic instability in the cultures is negligible (Withers 1985a).

Embryogenic cell suspension cultures have proven very useful for the isolation of protoplasts and genetic transformation studies (Vasil and Vasil 1979, 1980; Vasil *et al.* 1988, 1990; Potrykus 1990). They are normally obtained from callus cultures initiated from leaves, immature embryos and inflorescences. These callus and suspension cultures are routinely maintained by subculturing to fresh medium every month and every week, respectively. As formerly discussed, this is a time consuming and labor intensive process and can lead to genetic variation. The explants used to initiate the callus are not available throughout the year. It would thus be very useful if explants used to initiate the callus as well as the cell lines derived from them could be stored by cryopreservation, to be used whenever the need arises, and to serve as a permanent source of unique cells.

The objectives of this project are to study the factors involved in the cryopreservation of immature embryos, callus and cell suspension cultures of graminaceous species, and to regenerate plants from the cryopreserved tissues.

CHAPTER 2

LITERATURE REVIEW

Experimental work on freezing of biological material has been recorded from the mid 19th century. Significant progress, however, has been made only after the middle of this century (Withers and Street 1977a; Meryman and Williams 1985). Cryopreservation has proven to be a valuable tool for the storage of valuable genomes in microbiology, medicine and animal husbandry (Meryman 1966; Ashwood-Smith and Farrant 1980). Cryopreservation of plant germplasm is a more recent development that emerged both from studies of cold hardiness and freezing injury in plants (Levitt 1966; Li and Sakai 1978, 1982), and from the cryopreservation of animal cells and microbes.

Successful freeze preservation of plant cell cultures was first reported by Quatrano in 1968. A cell suspension of *Linum usitatissimum* L. (flax), stored at -50°C for up to one month using 10% (v/v) DMSO (dimethyl sulfoxide), retained a viability of only 14%, because the temperature employed was not low enough for stable long term storage.

True cryopreservation of in vitro cultures of higher plants was first reported in 1973 by Nag and Street, and Sakai and Sugawara, in two independent studies. Suspension cultures of carrot (*Daucus carota*) were frozen to liquid nitrogen temperature using 5% DMSO as a cryoprotectant, and survival rates of up to 68% (based on fluorescein diacetate test) were obtained. No decline in survival was observed even after 100 days of storage in liquid nitrogen, and when transferred to

an appropriate medium, healthy plants of normal morphology were recovered (Nag and Street 1973). Callus cultures of *Populus euramericana* cv. *gelrica*, cold acclimated prior to freezing, survived liquid nitrogen temperatures in the absence of cryoprotectants (Sakai and Sugawara 1973).

Freeze Injury

Different hypotheses have been put forward to help explain the mechanisms of cell injury during freezing. The two-factor hypothesis of Mazur *et al.* (1972) states that cell injury results from either the concentration of solution by extracellular ice, producing "solution effects" (i.e. solute concentration, changes in pH, and reduction in cell volume), or by formation of intracellular ice which causes mechanical injury to the plasmalemma and organelle membranes (Lovelock 1953; Lusena 1965; Mazur 1966; Sukumaran and Weiser 1972; Steponkus and Wiest 1978; Steponkus *et al.* 1982a; Hofmo and Berg 1989). It has also been suggested that alterations in membrane properties, rather than their mechanical breakdown, cause freezing injury (Heber 1967, 1968; Palta *et al.* 1977a,b; Palta and Li 1980). Palta *et al.* (1982) suggested that membrane proteins are the sites of membrane alterations while membrane lipids remain unaltered in spite of freezing injury.

According to the "minimum volume hypothesis," for every cell there is a minimal volume beyond which it cannot be reduced without injury (Meryman 1974). Any further volume decrease will cause stress in the membranes resulting in increased permeability, loss of membrane material, or irreversible mechanical disintegration (Meryman and William 1985).

Freeze damage can be prevented in tissues either by "freeze hardening," or by treatment with cryoprotective additives. The two methods have many aspects in common. Both involve a decrease in cellular water content and an increase in

specific solutes (Finkle *et al.* 1985b; Delvallee *et al.* 1989). Freeze hardened cells are no more resistant to osmotic dehydration than freeze sensitive ones, but they have evolved mechanisms to cool to lower temperatures before achieving the same degree of cell dehydration (Meryman *et al.* 1977; Steponkus and Wiest 1978; Meryman and William 1985). This is done in a variety of ways, including synthesis of additional intracellular solutes that increase the nonaqueous volume of the cell, "binding" of water, mechanical resistance to plasmolysis, and membrane leak (Williams and Meryman 1970; Williams and Williams 1976; Meryman *et al.* 1977).

Studies with cold-hardened and non-hardened rye mesophyll cells indicated that during extracellular freezing to lethal temperatures, membranes of non-hardened cells "roll up" and fuse to form multilayered vesicles, and eventually lose their phospholipid lamellar lattice altogether. No fusion, membrane roll up or loss of lamellar lattice was observed in cold-hardened cells (Singh and Miller 1985). Studies using protoplasts isolated from non acclimated and acclimated rye (*Secale cereale* L. cv Puma) leaves showed formation of endocytotic vesicles and exocytotic extrusions during freezing, respectively. During osmotic expansion following thawing the endocytotic vesicles remained in the cell and the protoplasts lysed before reaching their original volume (Gordon-Kamm and Steponkus 1984a), a phenomenon referred to as "expansion induced lysis" (Steponkus and Wiest 1978, 1979; Steponkus 1985a), whereas the exocytotic extrusions were drawn back into the surface of the protoplast thereby preventing lysis (Gordon-Kamm and Steponkus 1984b). In acclimated tissue freeze injury occurs at a lower temperature, predominantly by the complete loss of osmotic responsiveness following cooling (Steponkus *et al.* 1982a). These findings have been confirmed by direct cryomicroscopic observations (Dowgert and Steponkus 1984; Steponkus and Lynch 1989), and micro-osmotic manipulation (Dowgert *et al.* 1987)

According to Siminovitch and Levitt (1941), the plasma membrane of plants that survive very cold conditions is more resistant to damage by dehydration and mechanical breakdown. These differences are due to modifications in membrane composition, including substantial changes in the lipid composition of the plasma membranes during cold-hardening (Lynch and Steponkus 1987; Steponkus and Lynch 1989).

Freeze damage can also be reduced by the use of cryoprotective compounds which protect the cell from freeze injury by decreasing the freezing point of the cytoplasm, by reducing the size and growth rate of ice crystals, by protecting the cells against high intra- and extra-cellular solute levels and by stabilizing the membrane components (Withers 1980).

Cryopreservation of plant cell cultures includes a number of steps:

1. Pregrowing the cells in a medium supplemented with cryoprotectants or osmotic compounds (when appropriate).
2. Addition of cryoprotective compounds prior to freezing.
3. Freezing to ultra-low temperatures.
4. Storage of frozen cultures at liquid nitrogen temperatures.
5. Thawing of frozen cells.
6. Determination of viability.
7. Reculture on appropriate medium.
8. Induction of growth and regeneration of plants.

Pregrowth

Freeze tolerance can be increased prior to freezing either by cold-hardening (Sakai and Sugawara 1973; Seibert and Wetherbee 1977; Chen and Gusta 1982; Delvallee *et al.* 1989) or by growing the cells in a culture medium supplemented

with osmotic additives such as mannitol (Withers and Street 1977a,b; Pritchard *et al.* 1986), sorbitol (Chen *et al.* 1984a,b; Pritchard *et al.* 1986; Kartha *et al.* 1988), trehalose (Bhandal *et al.* 1985), various sugars (Latta 1971; Bannier and Steponkus 1972; Finkle *et al.* 1985b), proline (Withers and King 1979a,b) or cryoprotective compounds (Kartha *et al.* 1980, 1982). Osmotically active compounds probably act by inhibiting cell expansion, resulting in reduction in cell size, vacuolar volume and consequently water content (Withers and Street 1977b; Withers 1985b; Pritchard *et al.* 1986). Sixty percent reduction in vacuolar volume was observed when sycamore cells were grown in the presence of 6% mannitol for seven days (Pritchard *et al.* 1982).

Cryoprotection

Cryoprotective compounds can be of two types. Small molecular weight compounds such as DMSO, glycerol, methanol and acetamide penetrate the cells and act by colligative action causing a freezing point depression. Therefore, at a given temperature there is an increased volume of liquid available as solvent to minimize the deleterious action caused by electrolyte concentration due to dehydration during freezing (Finkle *et al.* 1985b).

Large molecular weight compounds such as polyvinylpyrrolidone (PVP), polyglycol, dextran, lactose and sucrose (Rowe 1966) do not penetrate the cell but act by osmotic action (Sakai 1962; Mazur 1970; Finkle *et al.* 1985b). Excess water is removed from the cell during the initial phases of cooling, particularly between -10°C and -20 °C (McGann 1978). If dehydration is carried out too far, accumulation of excess amounts of damaging solutes in the cell can cause "solution effects."

Disulfide bond formation, one of the reasons for frost injury (Levitt 1962), can also be prevented by cryoprotectants which inhibit the formation of these S-S bonds (Andrews and Levitt 1967). Cryoprotectants also interact with the cell membrane, either directly or indirectly, and stabilize its water-lipid-protein complex tertiary structure (Rowe 1966).

Most commonly used cryoprotectants are DMSO and glycerol. The successful use of DMSO as a cryoprotectant for animal cells was first reported by Lovelock and Bishop in 1959, but its use in the freezing of plant tissue was not reported until 1968 (Quatrano 1968). DMSO is an efficient cryoprotectant because of its low molecular weight, easy miscibility with water, lack of toxicity at low concentrations, rapid penetration and easy removal from the cells (Bajaj and Reinert 1977). Farrant (1972) reported that DMSO prevents cell damage during freezing by postponing both the shrinkage of the cell and the onset of cation leaks to higher osmolalities.

The optimal concentration of DMSO is found to be between 5-10% (Latta 1971; Nag and Street 1973, 1975a; Dougall and Wetherell 1974; Seibert and Wetherbee 1977; Sakai and Sugawara 1978; Sala *et al.* 1979). At high concentrations it is known to exert toxic effects by interfering with membrane permeability, and RNA and protein synthesis (Bajaj *et al.* 1970; Morris 1976; Barnett 1978; Zavala and Finkle 1980, 1981; Kartha *et al.* 1982; Finkle *et al.* 1985b). Concentrations ranging from 5% to 20% were found to exert the same level of toxicity on cells of *Picea glauca*, but towards the end of the regrowth period the cells recovered and a growth rate of 80-90% of untreated control was attained irrespective of the concentration used (Kartha *et al.* 1988).

Exposing cells to DMSO for long to medium term may also be damaging to the cells (Dougall and Wetherell 1974; Nag and Street 1975a; Morris 1980; Kartha *et al.* 1982). There is some indication that in higher plants DMSO protects cells

against genetic damage from radiation occurring at ultra-low temperatures (Ashwood-Smith 1967; Kaul 1970; Ashwood-Smith and Friedmann 1979; Finkle *et al.* 1985b; Withers 1988).

First successful use of glycerol was reported by Polge *et al.* in 1949. It has been used extensively for the cryopreservation of microbial and animal cells. Glycerol is not very effective in preventing freeze damage when used alone. Reduced uptake at the temperature of application could even cause injury by excessive plasmolyzation (Latta 1971; Nag and Street 1975a; Towill and Mazur 1976; Withers 1985b). It acts either as a penetrating compound or a non-penetrating compound depending on the temperature of application (James 1983). The optimal concentration of glycerol when used alone is 5-10% (Latta 1971; Nag and Street 1975a,b).

Using a mixture of cryoprotectants at low concentrations is better than using a single cryoprotectant at higher concentrations. An additive cryoprotective effect of the combined compounds is obtained while the concentration of toxic compounds is reduced (Finkle and Ulrich 1978, 1979; Hauptmann and Widholm 1982; Withers 1982, 1985b; Bajaj 1983; Chen *et al.* 1984a,b; Finkle *et al.* 1985b; Kartha *et al.* 1988). Combinations of cryoprotectants are used at a total concentration of 0.5-2 M (Withers 1980). Cryoprotectants are applied to cells during the period immediately preceding cooling. They are normally prepared in culture medium except in a few instances where they were dissolved in water with or without sugar (Uemura and Sakai 1980; Watanabe *et al.* 1983), and the pH is adjusted to the standard pH of the medium used. Because of the high concentrations of solutes, such mixtures are prone to caramelization if autoclaved, hence they should be sterilized by filtration (Withers 1986).

Cold application (0°C) of cryoprotectants is beneficial over application at room temperature. Toxic effects of the cryoprotectants are probably reduced due to

slower metabolic activities of the cells at lower temperatures (Finkle and Ulrich 1982). Sudden mixing of the cryoprotectant with the specimen may lead to damage by plasmolysis, therefore the cryoprotectants should be added to the culture gradually over a period of time (Nag and Street 1975a; Towill and Mazur 1976; Withers and Street 1977a,b; Bajaj 1979a). Cryoprotectants are added at a reduced temperature (0-4°C), since an exposure to the cryoprotectants at room temperature has adverse effects on the tissue.

Freezing

Cells can be frozen by slow freezing, stepwise freezing, and rapid freezing. Freezing as previously mentioned can cause injury due to "solution effects" caused by extracellular freezing, or mechanical damage caused by intracellular freezing (Mazur *et al.* 1972). Intracellular freezing is lethal and to prevent this cooling should be such that all freezable water flows out of the cell before intracellular freezing ensues, and yet rapid enough to prevent solution effects (Mazur 1970; Bajaj 1979a). Slow cooling is the most widely used procedure especially for cryopreservation of cell suspensions and protoplasts (Withers and Street 1977a; Chen *et al.* 1984b). However, too slow a rate of cooling can be damaging due to excessive dehydration which results in solution effects (Mazur 1969, 1970; Meryman *et al.* 1977; Withers 1984a). Cooling rates of 0.5°-4°C/min give best results (Withers and Street 1977a), with the optimum at around 2°C/min (Dougall and Wetherall 1974; Henshaw 1975; Nag and Street 1975b; Bajaj 1976a,b).

Stepwise freezing is achieved by exposing the specimens to one or more intermediate temperatures (Sugawara and Sakai 1974; Sakai and Sugawara 1978). At -30°C all freezable water is removed from the cell due to extracellular freezing, and they are not injured by exposure to extremely low temperatures (Sakai 1960).

This temperature may vary with the degree of frost-hardiness (Sakai 1965). A combination of slow and stepwise freezing, in which the specimen is cooled at a slow rate to ultra-low holding temperatures, and held for up to one hour at this temperature before plunging into liquid nitrogen, has been found to be very useful (Farrant *et al.* 1977; Withers 1985b). Heszky *et al.*(1990) reported that cell survival could be considerably improved by holding suspension cultures of *Puccinellia distans* (L.) Parl at sub-zero temperatures for 10-30 minutes before plunging into liquid nitrogen. Cooling to too low or too high a transfer temperature or prolonged exposure to suboptimal temperatures before transfer to liquid nitrogen, may be injurious due to excessive cellular dehydration and intracellular freezing (Withers 1985c; Kartha *et al.* 1988).

Rapid freezing at rates up to >1000°C/min is achieved by plunging the specimen into liquid nitrogen directly. This is not recommended for cell suspensions (Bajaj 1976a; Kartha 1985b), but has been successful for organized structures (Seibert 1976). Even though intracellular ice crystals are formed, they do not have enough time to grow to damaging sizes because of the high rates of cooling (Bhojwani and Razdan 1983; Withers 1984a). Also, during rapid thawing they melt before attaining damaging sizes (Sakai and Sugawara 1973; Sugawara and Sakai 1974). Harmful effects of ice crystals are determined by the amount, crystal size and location of the ice (Withers 1987). Farrant *et al.* (1977) reported that it is the amount rather than the size of intracellular ice crystals that determines survival during thawing. Rapid freezing is the method of choice for pollen and meristems which have very low water content and are adversely affected by solution effects during slow freezing (Nath and Anderson 1975; Withers 1978a). Withers (1979) used a dry freezing method for cryopreservation of clonal plantlets of *Daucus carota*, where the specimens were blotted dry and enclosed in foil envelopes.

Presterilized, polypropylene screw cap ampoules of different capacity are used for freezing of the samples. Flame sealed glass ampoules are not recommended because incomplete sealing could result in liquid nitrogen penetration during storage and explosion upon thawing (Withers 1986).

Storage

The storage temperature should be low enough to stop all metabolic activity and prevent biochemical injury. Storage at higher temperatures causes structural damage to cells as a result of progressive recrystallization of ice, resulting from the migration of water in the solid state (Withers 1985b). Temperatures in the range of -4°C to -70°C are not recommended for long term storage, since deterioration of the cells is observed at such temperatures (Bajaj 1983; Withers 1984a). At -20°C protein denaturation takes place because of metabolic activity, leading to death of cells. In addition, changes in concentration of solutes and subsequent variation in pH also take place at these temperatures (Bajaj 1979b).

For short to medium term storage, a temperature of -80°C may be adequate. Although intracellular ice is maintained at this temperature, structural damage and consequent progressive deterioration in viability occurs due to recrystallization of ice (Nag and Street 1973; Nei 1973; Withers 1980, 1986). Recrystallization of ice cannot be prevented when storage temperatures above -130°C are used (Henshaw 1975; Bhojwani and Razdan 1983), hence for long term storage, temperatures of liquid nitrogen are recommended. Liquid nitrogen and suitable storage containers are available commercially, and a container holding about 4000, 2 ml capacity ampoules normally consumes 20 - 25 l of liquid nitrogen per week (Withers and Street 1977a).

Even though metabolic activity is completely stopped at liquid nitrogen temperatures, molecular changes due to ionizing effect of radiation may lead to cumulative damage (Withers 1987), but this may not pose a major problem at least for decades of storage (Whittingham *et al.* 1977).

Thawing

Thawing can be achieved either rapidly at rates of 500-750°C/min by plunging the frozen sample into water at 37-40°C (Chen *et al.* 1984b), or slowly by exposing the vials containing the specimens to blown warm air, air at room temperature, or to liquid nitrogen vapor followed by air at room temperature (Sakai and Sugawara 1973; Dougall and Wetherell 1974; Withers 1978b, 1979, 1980, 1983). Contents of the ampoule should be thoroughly mixed to prevent local heating while thawing proceeds and the ampoule should be transferred to an ice bath as soon as the ice plug in each ampoule disappears, which could be anywhere from 60-90 seconds when thawed rapidly, and would take up to 20 minutes when thawed slowly (Withers 1980).

Recrystallization of ice crystals occurs during slow thawing, particularly at temperatures above -45°C, resulting in the formation of progressively larger ice masses which cause damage to cell membranes and cytoplasmic organelles. This zone of recrystallization is passed so rapidly during rapid thawing that the ice crystals melt before they have the opportunity to recrystallize (Sakai and Otsuka 1967; Sakai and Yoshida 1967; Bajaj and Reinert 1977).

Rapid thawing is more advantageous than slow thawing only at higher transfer temperatures. When specimens are cooled to temperatures beyond a transfer temperature of -40°C, the thawing rates do not have any effect on recovery

rates (Withers 1985b), probably because of the reduction of water content of the cell to an optimal level (Bhojwani and Razdan 1983).

Recovery

Thawed cultures can be recovered by three methods:

1. Removing the cryoprotectants by washing before returning to culture.
2. Diluting the cryoprotectants out by using liquid medium.
3. Inoculating into semi-solid medium directly without washing.

Post-thaw washing was routinely used in early studies to avoid deleterious effects of cryoprotectants (Latta 1971; Nag and Street 1973; Dougall and Wetherell 1974; Withers and Street 1977b; Sala *et al.* 1979; Kartha *et al.* 1982). Stepwise dilution may be advantageous for avoidance of deplasmolysis injury (Towill and Mazur 1976; Withers and Street 1977a). The temperature of the washing solution is important in the survival of the specimen, and 0°C is generally employed (Kartha *et al.* 1982). Sugarcane and rice cultures showed a higher viability when washed at room temperature than at 0°C, probably due to the differences in membrane fluidity at these temperatures (Finkle and Ulrich 1982). Recent studies have shown that washing may be more deleterious than prolonged exposure (Withers and King 1979b; Chen *et al.* 1984b). Freezing and storage at low temperatures could result in loss of water, ions, sugars and amino acids due to increased membrane permeability (Siminovitch *et al.* 1964; Lyons 1973; Palta *et al.* 1977a,c). Washing or diluting with liquid medium would result in removal of these compounds which may be required for the recovery of the thawed cells (Withers 1979; Withers and King 1979b). Washing or diluting may also result in deplasmolysis injury resulting from the loss of membrane material during freeze dehydration (Wiest and Steponkus 1978; Withers and King 1979b; Withers 1985c). Supplementing the washing medium with an

osmoticum such as mannitol or sorbitol, and removing the cryoprotectants by stepwise dilution have been employed to minimize deplasmolysis injury (Towill and Mazur 1976; Withers and Street 1977a; Maddox *et al.* 1983).

Placing the cells without washing on semi-solid medium similar to the composition of the liquid medium, has been found to be better than washing or diluting with the culture medium, by allowing the toxic cryoprotective compounds to gradually diffuse out (Withers and King 1980; Finkle *et al.* 1985b).

Frozen and thawed cells, when returned to culture, may start to grow within a day or two (Sala *et al.* 1979), or enter a prolonged lag phase of even up to 1-6 months (Nag and Street 1973; Bajaj 1976a). This could be due either to the revival and repair that take place in the cold shock and partially damaged cells or due to the suppression of growth by the cryoprotectants (Bajaj and Reinert 1977). Cella *et al.* (1982) reported that freeze-thawed cells of rice showed a number of physiological alterations, including reduction in respiration and glucose uptake, loss of intracellular ions, decrease in cellular levels of key metabolites and fragility of the protoplast, all of which were repaired during a lag period of 2-4 days. Cells may fail totally to enter growth if the viable cell density falls below the minimum inoculum level (Stuart and Street 1969, 1971; Maddox *et al.* 1983).

Determination of viability

Viabilities of cryopreserved and thawed cells can be determined by various means. For a reliable estimation of viability, growth parameters such as mitotic index, cell number, packed cell volume, increase in fresh and dry weights and plating efficiencies should be employed. For rapid processing, and to screen large numbers of specimens, growth parameters cannot be used. In such instances, staining

reactions are employed for the determination of viability, especially for cell suspensions.

TTC (2,3,5-triphenyl tetrazolium chloride) test involves the reduction of tetrazolium salts by mitochondrial activity, to a water insoluble red compound, formazan. Formazan can be extracted in ethanol and examined spectrophotometrically (Steponkus and Lanphear 1967; Towill and Mazur 1975). The amount of formazan produced is proportional to the number of viable cells present, and can be used to estimate viability of cells (Towill and Mazur 1975).

Flourescein diacetate test, developed by Widholm (1972), is based on the ability of viable cells to uptake and break down flourescein diacetate by esterase activity. Only the viable cells can be visualized under UV light by their fluorescence (Withers 1984b). Cells that emit intermediate levels of florescence and cell aggregates where the central cells cannot be clearly seen are complicating factors for this method (Withers 1980).

Evans blue staining is based on the ability of the viable cells to exclude a dye (Gaff and Okong'O-Ogola 1971). Cells are treated with a dye and observed after a few minutes. The viable cells remain colorless and the dead cells show blue coloration. The same principle applies for phenosafranine staining which is also used for the estimation of viability of cell suspensions (Widholm 1972). The drawback of these methods is that if left too long in the stain all the cells become colored (Bajaj 1979a).

Staining reactions should not be used as the sole means of estimation of viability, since there may be cells present which show positive reaction immediately after thawing but later die in culture, or cells in a state of cold shock giving a negative reaction but later reviving in culture (Bajaj 1976a, 1979a).

Cryopreservation of Cell Suspension Cultures

Cell suspension cultures are widely used in physiological studies and for plant regeneration, protoplast isolation, genetic transformation, and secondary product synthesis. Cryopreservation has been used successfully for the storage of cell suspensions which cannot be achieved by slow growth (Rose and Martin 1975).

Age, nature and the physiological state of cells considerably influence their survival during cryopreservation (Bajaj 1979a, 1983). Generally, with cell suspensions, small non-vacuolated thin walled cells survive better than larger highly vacuolated thick walled cells (Nag and Street 1975a; Bajaj 1976a; Withers and Street 1977b).

Cells in the late lag or early exponential phase are more resistant to freeze damage (Sugawara and Sakai 1974; Withers and Street 1977b; Sakai and Sugawara 1978). A decrease in viability is generally observed in stationary phase cells (Sala *et al.* 1979), probably due to the higher vacuolation and water content associated with increased cell size and consequently a smaller surface area to volume ratio, which impede protective dehydration (Withers and Street 1977b; Withers 1978c). Lag and stationary phases can be effectively eliminated by rapid subculture, which also causes a reduction in cell size (Withers and Street 1977a; Withers 1978c). Mitotic stage also influences the survival of suspension cultures (Withers 1985b). Experiments on synchronized cultures of *Acer pseudoplatanus* by nitrate starvation revealed that cells in G_1 or G_0 phase were more tolerant to freeze damage, while the highest viability loss was observed during cytokinensis or S phase (Withers 1978c). Synchronization and selection of suitable cell stage is not recommended in cryopreservation of cell suspensions, because of the relatively brief periods of freeze tolerance and the technical demands involved.

Filtered cell suspensions consisting of free cells showed poor survival compared to actively growing suspensions consisting of highly cytoplasmic cells in

small colonies (Bajaj 1976a). Survival is also reduced in large aggregates where the proportion of highly meristematic, rapidly dividing cells is reduced (Withers 1983). The location of the cells in an aggregate also determines their survival, the central cells being more susceptible due to differences in freezing rates, and the degree of protective dehydration (Withers and Street 1977a; Withers 1978b; Withers 1983).

Cell suspensions are commonly grown for a minimum of 3-4 days in a medium supplemented with osmotic additives to increase freeze tolerance prior to freezing (Withers 1983, 1984b, 1985b). Mannitol is the most widely used pregrowth additive for cryopreservation of cell suspensions, and concentrations up to 6% (v/v) have been successfully used (Withers 1985b). Mannitol did not enhance the survival of *Nicotiana sylvestris* cells whereas the closely related compound sorbitol increased their survival considerably (Maddox *et al.* 1983). Pregrowing the cells in the presence of cryoprotective additives such as DMSO is found to be of little value for cryopreservation of cell suspensions (Withers 1983).

For cryopreservation of cell suspensions, the cryoprotectants are prepared in double strength and normally added gradually to an equal volume of cell suspension at low temperatures. The cell suspension is often concentrated before cryoprotectant addition to provide sufficient cell density during recovery (Stuart and Street 1969; Sala *et al.* 1979). Mixtures of cryoprotectants are generally more successful than single compounds. Mixtures of 0.5 M DMSO, 0.5 M glycerol and 1 M sucrose or proline adapted by Withers and King (1980), sorbitol and DMSO by Chen *et al.* (1984a,b), or glycerol and DMSO (Nag and Street 1975a,b; Hauptmann and Widholm 1982; Maddox *et al.* 1983), are some of the combinations commonly used.

Slow freezing to sub-zero temperatures and then immersion in liquid nitrogen is the most widely used mode of freezing for cryopreservation of cell suspensions. Rapid freezing is not recommended. Rapid thawing is found to be

more beneficial than slow thawing (Sugawara and Sakai 1974; Nag and Street 1975b; Seibert and Wetherbee 1977; Withers and Street 1977a; Withers 1978b; Withers and King 1980; Kartha *et al.* 1982). Dougall and Wetherall (1974) reported that slow thawing could be used successfully for cell suspensions of carrot, but the viability was never more than that with rapid thawing.

Staining reactions are commonly employed for a quick estimation of viability. TTC test is found to be reliable for cell suspensions (Sugawara and Sakai 1974; Towill and Mazur 1976; Finkle and Ulrich 1979), although there are reports in which TTC test results could not be faithfully correlated with estimates obtained from other tests (Bajaj and Reinert 1977; Withers 1980; Sala *et al.* 1979).

Flourescein diacetate test, another commonly used staining reaction for cryopreserved cell suspensions, has been found to be reliable in some studies (Nag and Street 1975a; Withers and Street 1977a). However, unreliability by over-estimation (Pritchard *et al.* 1986) as well as under-estimation (Withers and Street 1977b) have been documented.

Cell are returned to semi-solid medium rather than liquid medium for rapid recovery (Withers and King 1979b). Washing is not recommended and the potentially damaging cryoprotectants can be gradually diluted out by plating the cells on a filter paper layered on a semi-solid culture medium, and transferring to fresh medium after a period (Chen *et al.* 1984b).

Cryopreservation of Callus Cultures

Short term storage of callus cultures by slow growth has been attempted by both reduction in temperature (Bannier and Steponkus 1972, 1976; Hiraoka and Kodama 1982, 1984), and reducing the availability of oxygen by mineral oil overlay (Caplin 1959; Bridgen and Staby 1981; Augereau *et al.* 1986). In some strains, the

morphogenetic capacity and secondary metabolite production were retained during storage by slow growth, whereas in others they were lost (Hiraoka and Kodama 1982, 1984).

Cryopreservation of callus cultures is not as widely explored as cryopreservation of cell suspensions, and only limited success has been reported. Callus cultures have the same requirements for cryopreservation as cell suspensions, but they are less amenable to freezing, because protective dehydration and cryoprotectant penetration are impaired by the size and heterogeneity of callus tissue (Withers 1980). Fractionating the callus into small pieces before freezing has been employed in some cases to avoid this problem (Watanabe *et al.* 1983).

Unlike suspension cultures, callus cultures are generally not pregrown on medium supplemented with cryoprotective additives or high osmoticum, because of their growth habit (Withers 1986). Pregrowth treatment involving cold acclimation is also not commonly employed, but has been found to be useful for cryopreservation of callus cultures of *Populus euramericana* cv. *gelrica* in the absence of cryoprotectants (Sakai and Sugawara 1973).

Combinations of cryoprotectants such as 10% polyethylene glycol, 8% glucose and 10% DMSO (Ulrich *et al.* 1979, 1984a,b; Finkle *et al.* 1980, 1983, 1985a,b; Tisserat *et al.* 1981; Finkle and Ulrich 1982), 0.5 M sorbitol and 5% DMSO (Chen *et al.* 1985), 0.5 M DMSO, and 0.5 M glycerol with 1 M sucrose (Hahne and Lorz 1987) have been successfully used.

Slow freezing at rates from 0.5 - 3°C/min to sub-zero temperatures, followed by storage in liquid nitrogen, rapid thawing and reculture with or without washing is the routine method used (Ulrich *et al.* 1979, 1984b,c; Chen *et al.* 1985). Rapid cooling by direct immersion into liquid nitrogen was reported to be deleterious (Ulrich *et al.* 1979; Hahne and Lorz 1987). Blotting dry the specimen before freezing and omitting the washing after thawing improved the survival rates

considerably (Hahne and Lorz 1987). Viability is determined by regrowth potential. Staining reactions are generally not employed (Ulrich *et al.* 1979, 1984b,c; Tisserat *et al.* 1981; Chen *et al.* 1985; Hahne and Lorz 1987). A short lag period is observed in some cases (Tisserat *et al.* 1981; Hahne and Lorz 1987) and retention of morphogenetic and biosynthetic capacity after cryopreservation have been documented (Tisserat *et al.* 1981; Watanabe *et al.* 1983; Ulrich *et al.* 1984c; Chen *et al.* 1985; Hahne and Lorz 1987).

Cryopreservation of Protoplasts

Freezing of protoplasts is mainly used as a model system to investigate the effects of freezing and thawing on the structure and function of the plasma membrane in the absence of complicating factors from the cell wall (Singh 1977, 1979a; Wiest and Steponkus 1977a,b, 1978; Siminovitch *et al.* 1978; Steponkus and Wiest 1978; Levin *et al.* 1979; Bartolo *et al.* 1987). They have also been used for long-term storage of germplasm (Takeuchi *et al.* 1982). However, because of the difficulties encountered in regenerating plants from protoplasts of most species, the usefulness of protoplasts for germplasm conservation is negligible. Cell walls of protoplasts begin to regenerate within minutes of culture, and to prevent this, the method employed for storage should be such that all metabolic activity is at a standstill.

Isolated protoplasts are suspended in a medium supplemented with an osmotic stabilizer, and cryopreservation usually follows immediately to prevent the regeneration of cell wall (Withers 1985b), except in one study where a period of equilibration was required (Mazur and Hartmann 1979). Requirements for successful cryopreservation of protoplasts are similar to those of cell suspensions, but they cannot be exposed to any pregrowth conditions because of their delicate

and ephemeral nature (Withers 1985b, 1986). A method involving cryoprotection with a single or a mixture of cryoprotectants, followed by freezing at 1-2°C/min to -40°C, storage in liquid nitrogen and rapid thawing has been used for cryopreservation of plant protoplasts (Hauptmann and Widholm 1982; Takeuchi *et al.* 1982; Withers 1983). Freezing the protoplasts in heat sealed foil envelopes improved the viabilities considerably, probably due to uniform freezing rates obtained from better heat conductivity (Takeuchi *et al.* 1980).

No special post-thaw treatment is required (Withers 1983), and a quick estimation of viability can be obtained from staining reactions (Hauptmann and Widholm 1982). Cell wall regeneration could be observed within a few hours by using the fluorescent dye, Calcofluor White. Cryopreserved protoplasts retained their morphogenetic capacity (Takeuchi *et al.* 1982).

Cryopreservation of Shoot-Tips

Shoot-tip cultures, commonly used for elimination of viruses (Ingram 1973; Kartha and Gamborg 1975; Walkey 1976; Grout *et al.* 1978; Kartha 1985a), are ideal for the conservation of vegetatively propagated plants (Henshaw 1975; Morel 1975; Bajaj 1979a; Kartha 1985a). They are genetically more stable than either callus or suspension cultures, and thus eminently suited for germplasm storage and conservation (D'Amato 1975).

Storage of shoot-tips by slow growth has been successful for short to medium term, but long term storage requires periodic renewal (Mullin and Schlegel 1976; Lundergan and Janick 1979; Monette 1986). Cryopreservation of shoot-tips is more difficult, because of the diversity of the cells and the need to maintain their organization (Henshaw 1982).

The first report of cryopreservation of shoot-tips was by Seibert in 1976. Aseptically excised shoot-tips of *Dianthus caryophyllus* were frozen to -196°C using 5% DMSO as cryoprotectant, and viabilities up to 33% were obtained. Cryopreservation of shoot-tips of in vitro maintained plantlets was first reported by Kartha *et al.* (1980).

Prior to freezing, the shoot apical meristem region, which is amenable to freezing, should be excised from the shoots which in general cannot be frozen intact (Withers 1985c). The shoot meristem at the early stages of development is best for survival (Benson *et al.* 1989), and 0.5-1 mm specimens consisting of the apical dome and 2-4 intact leaf primodia are ideal for cryopreservation (Seibert and Wetherbee 1977; Grout and Henshaw 1978).

Shoot-tips are normally frozen after a pregrowth period of 1-3 days after excision (Grout and Henshaw 1978, 1980; Haskins and Kartha 1980; Kartha *et al.* 1980; Henshaw *et al.* 1985; Benson *et al.* 1989). The increase in viability after pregrowth can be attributed to the healing of the dissection wound and/or the achievement of an optimum unit size (Withers 1985c). Supplementing the pregrowth medium with nontoxic levels of DMSO improved the percentage of recovery (Kartha *et al.* 1979, 1980; Manzhulin *et al.* 1983; Kartha 1985a). Cold-hardening is also employed to enhance survival to freezing (Seibert and Wetherbee 1977). Reed (1989) reported that by in vitro cold-hardening of plants of *Vaccinium corymbosum* for three or more weeks prior to excision, survival can be increased from 6% to 58%. However, Kartha *et al.* (1979) reported that freeze hardening at 4°C prior to freezing was not as satisfactory as pregrowing them in the presence of cryoprotectants.

DMSO at 5-15% is generally employed for cryopreservation of shoot-tips (Seibert 1976; Seibert and Wetherbee 1977; Grout and Henshaw 1978, 1980; Grout *et al.* 1978; Kartha *et al.* 1979; Haskins and Kartha 1980; Kartha 1984; Withers *et al.*

1988; Benson *et al.* 1989), whereas glycerol has been found to be inappropriate (Kartha *et al.* 1979; Grout and Henshaw 1980). Combinations of cryoprotectants have also been found to be useful (Bajaj 1982). Shoot-tips of apple (*Malus domestica* Borkh. cv. Fuji) survived liquid nitrogen temperatures following prefreezing to sub-zero temperatures in the absence of any cryoprotectant treatment (Katano *et al.* 1983).

Slow (Kartha *et al.* 1979, 1980, 1982; Towill 1981), intermediate (Seibert and Wetherbee 1977), and rapid cooling have been successfully used for the cryopreservation of shoot-tips (Grout and Henshaw 1978, 1980). Rapid cooling achieved by immersing the shoot-tips directly in liquid nitrogen with the aid of a hypodermic needle was used for the cryopreservation of shoot-tips of *Solanum goniocalyx*, while slow and intermediate cooling rates were ineffective (Grout and Henshaw 1978, 1980).

Rapid thawing is generally employed for cryopreserved shoot-tips (Grout *et al.* 1978; Grout and Henshaw 1980; Kartha *et al.* 1979; Haskins and Kartha 1980; Kartha 1984; Benson *et al.* 1989). The most important aspect after thawing seems to be the composition of the medium, and post-thaw washing seems to have no deleterious effects (Grout *et al.* 1978; Haskins and Kartha 1980; Withers 1985c). Post-freezing recovery was found to be significantly affected by alternating light conditions during culture (Benson *et al.* 1989), and the medium used in the recovery phase (Withers *et al.* 1988).

Microscopical studies show evidence of damage in randomly scattered sites composed of single cells or small groups of cells in the shoot-tip, but their presence did not have any detrimental effect on the developmental potential of the shoot primodium (Grout and Henshaw 1980). The survival of the dome entirely or partly after freezing is not essential for regeneration of plants from the shoot-tip, as surviving cells located laterally on the dome and on the shoot apical meristem have

the capacity to form shoot meristems (Haskins and Kartha 1980). Plantlets have been produced from cryopreserved shoot-tips of many species (Kartha *et al.* 1979, 1980; Grout and Henshaw 1980; Haskins and Kartha 1980).

CHAPTER 3

ULTRASTRUCTURAL STUDIES USING A CELL SUSPENSION OF *PANICUM MAXIMUM* TO DETERMINE THE CHANGES THAT OCCUR DURING THE PROCESS OF CRYOPRESERVATION

Introduction

Successful cryopreservation of plant tissue cultures have been reported in the past decade. However, the mechanism of freeze injury is far from understood. To optimize the technique of cryopreservation in such a way that it can be applied to any type of culture with high survival rates, it is important that the factors controlling freeze injury be well understood.

Membranes are known to be a major cause of freeze injury which can manifest in many different forms such as intracellular ice formation, loss of osmotic responsiveness during cooling, expansion-induced lysis during warming and altered osmometric behavior (Steponkus 1984, 1985a,b; Steponkus *et al.* 1982a,b).

More than 90% of *Panicum maximum* suspension culture cells can withstand ultra-low temperatures when pregrown in MS2C medium supplemented with 6% mannitol, and cryoprotected with 0.5 M sorbitol and 5% DMSO before being subjected to sub-zero temperatures (See chapter 4). In the absence of any cryoprotective treatment, cells did not survive the freezing process. Therefore, cells from the above mentioned treatments were used to study the differences at the ultrastructural level that enable the cells to withstand such cold temperatures.

Materials and Methods

Cell suspension culture of *Panicum maximum* was used to determine ultrastructural changes that occur during different stages of cryopreservation. The suspension was pregrown in MS (Murashige and Skoog 1962) basal medium supplemented with 3% sucrose, 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 100 mg/l inositol and 5% coconut water (MS2C medium) at weekly intervals. The pH of the medium was adjusted to 5.8 before autoclaving. Prior to freezing the suspension was pregrown in MS2C medium supplemented with 6% (w/v) mannitol for three days, treated with an equal volume of pre-chilled, filter sterilized cryoprotectant mixture composed of 10% DMSO and 1 M sorbitol, and cooled in a Cryomed 1010 Micro Computer Programmable Freezer Unit at a rate of 0.5°C/min to -40°C before transfer to liquid nitrogen. Cells not subjected to pregrowth or cryoprotectant treatment were also frozen as above, as well as by direct immersion into liquid nitrogen.

Rapid thawing was employed in all cases, where the ampoules containing the cells were removed from liquid nitrogen and dropped directly into a water bath at 40°C and swirled until the ice melted. The cells were then plated without washing on semi-solid MS2C medium for recovery.

Cells were fixed at the following stages for light and electron microscopical studies.

1. Three day old control cells without any pregrowth or cryoprotective treatments.
2. After three days in liquid MS2C medium supplemented with 6% mannitol.
3. After treatment with the cryoprotective additives.
4. Immediately after thawing.

5. Two days after thawing.
6. Ten days after thawing.
7. Cells frozen rapidly without any pregrowth or cryoprotectant treatments, thawed and fixed immediately.

The cells were fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer for two hours at room temperature, and then rinsed three times with 0.1 M sodium cacodylate buffer for a total of one hour. They were then treated with a secondary fixative consisting of 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for two hours at room temperature. Cells were rinsed with deionized water for a total of one hour to remove the osmium.

The cells were then dehydrated gradually by treating with 25%, 50%, 75%, 95% and 100% ethyl alcohol for twenty minutes each, and then transferred to a mixture of 1:1 absolute ethanol:absolute acetone for 30 minutes followed by another 30 minutes in 100% acetone. All dehydration steps were carried out at 0-4°C.

Cells were infiltrated in Spurr's resin (Spurr 1969). They were treated overnight with 30% plastic and 70% acetone, followed by 70% plastic and 30% acetone, and finally with 100% plastic. The cells were then transferred to fresh 100% plastic, poured into plastic molds and left overnight in a 60°C oven for polymerization. All infiltration steps were carried out at room temperature.

Cells with and without any cryoprotective treatments were cooled at a rate of 0.5°C/min and freeze-fixed according to the method described by Mackenzie *et al.* (1975), and adapted by Singh (1979b). A mixture of 2% osmic acid and 18% NaCl was prepared and stored at its equilibrium freezing point of -10°C. Suspension cultures of *Panicum maximum* with and without cryoprotectant treatments were transferred to polypropylene ampoules with their bases cut off and replaced with Parafilm. Excess water was removed and the ampoules were cooled to -10°C at a rate of 0.5°C/min. The cells were then transferred into the osmic acid mixture by

removing the Parafilm base, and stored at -10°C overnight. The osmic acid mixture was then removed and the cells were washed with three changes of distilled water over a one hour period. Dehydration and embedding were done next, as described previously.

For light microscopy semi-thin sections were cut on a Sorvall MT2-B ultra-microtome with a DuPont diamond knife, and the sections were picked up and placed on a drop of water on a gelatine coated slide prepared according to Hayat (1981). A drop of xylene was added to stretch the sections and the slide was kept on a hot plate at 60°C until the xylene evaporated. The water was then removed carefully by using a piece of tissue paper. A drop of toluidine blue in 0.1% NaCO3 at pH 11.1 (Trump *et al.* 1961) was added next and the slide was left on the hot plate until a golden ring was observed along the edge of the drop of stain. The specimen was then rinsed with water, dried and dry mounted with permount. Cells were observed under a Zeiss light microscope under normal and phase contrast lenses.

For electron microscopy thin sections were picked up on formvar coated (0.35%), 100 or 200 mesh grids, and post stained with 1% uranyl acetate (aqueous) for 15 minutes followed by lead citrate (Reynolds 1963) for 5 minutes. A Joel 100-CX transmission electron microscope operating at 60 KV was used for taking photographs of the specimens.

Differences in vacuolar volume as well as differences in sizes of the organelles were determined at different stages of the cryopreservation procedure by using morphometry as described by Toth (1982). The volume of a single mitochondrion in cells subjected to different treatments was compared by using point counting and then dividing the values obtained by the total number of mitochondria found in each treatment. The volume and area of the ER were determined in relation to the volume of the cell between different treatments. The number of interceptions per unit length of test line (N_L) was determined for each

cell. The surface area to volume ratio of the ER volume was obtained from the equation $4 \times N_L$ (Toth 1982).

Results

When observed under light and electron microscopes, the control tissue contained cells with a large nucleus and a few large vacuoles. Organelles such as mitochondria, golgi, amyloplast with starch granules and ER were clearly visible. Oil globules were also present in the cytoplasm (Figs. 1-5). The plasma membrane was relatively smooth (Figs. 6, 7), or had minor undulations (Fig. 8).

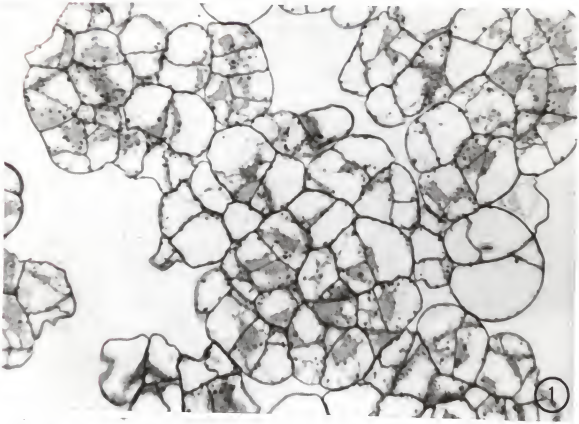
Cells pregrown in mannitol were smaller in size with dense cytoplasm. The cells either lacked a vacuole or the central vacuole was replaced by numerous smaller ones (Figs. 9, 10). The plasma membranes differed markedly from those of the control cells. Invaginations were frequently found in the multivacuolated cells (Figs. 11-14). The average number of invaginations found in one section was five. In some sections up to 12 invaginations could be observed. These invaginations were completely absent in control cells as well as cells fixed at all other stages of the cryopreservation procedure. A reduction in cell size was also observed in cells pregrown in mannitol, the volume being 89% of the control cells. Vacuolar volume was also considerably reduced in these cells. The vacuole of the control cells on average occupied 58% of the cell volume, whereas in mannitol pregrown multivacuolated cells it occupied only 23% of the cell volume.

Dilation of organelles also occurred in mannitol pregrown cells. Average volume of the mitochondria increased by 8%. The ER occupied 1% of the volume of the control cell, whereas in mannitol pregrown cells the volume of the ER was 1.2% in relation to the volume of the cell. The area of the ER in control cells was

Figs. 1 and 2. Groups of cells from a three day old suspension of *Panicum maximum*, observed under a light microscope. Cells are large and highly vacuolated.

1. x 280

2. x 200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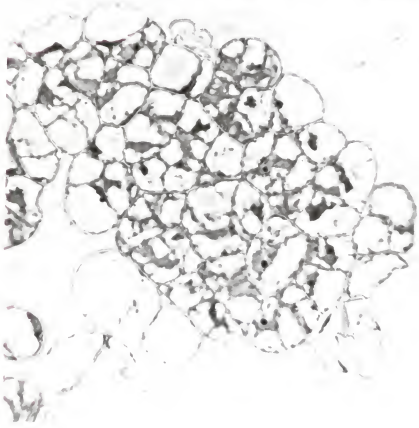
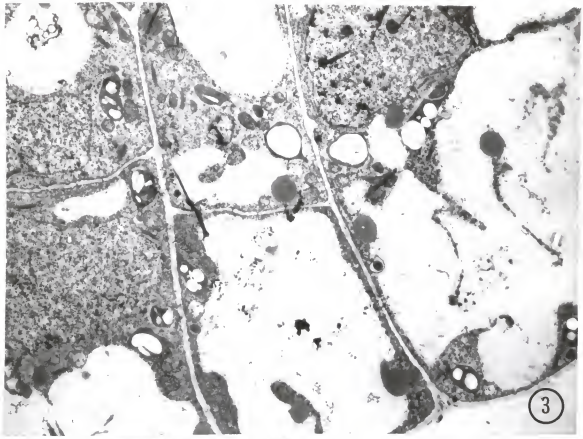

2

Figs. 3 and 4. Electron micrograph of a three day old suspension culture of *Panicum maximum* observed under an electron microscope showing nuclei, large vacuole, plastics with starch granules, mitochondria, ER and golgi.

3. x 8.5K

4. x 13K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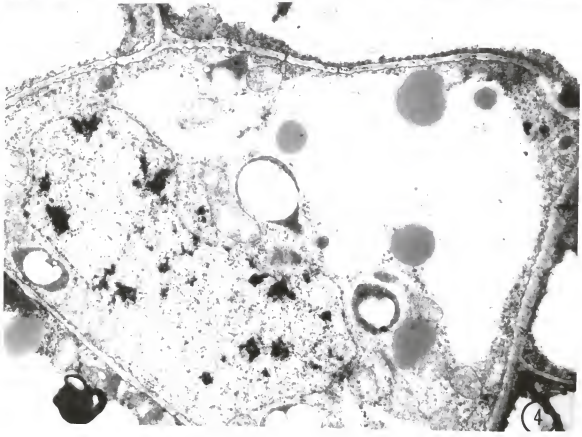
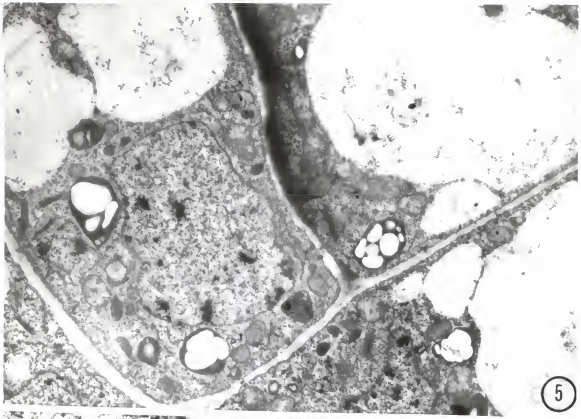

4

Fig. 5. Cells from a three day old suspension showing the presence of nucleus, large vacuole and organelles. x 10K

Fig. 6. Plasma membrane of a control cell showing the relatively smooth appearance. Mitochondria with cristae, plastids with starch granules, ER and oil globules are also seen. x 26K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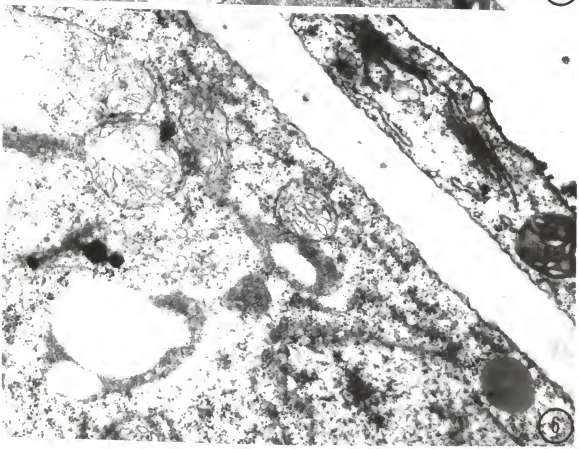

5

Figs. 7. Plasma membrane of a control cell showing smooth appearance. Organelles are also clearly seen. x 32K

Fig. 8. Plasma membrane of control cells with minor undulations. x 13K

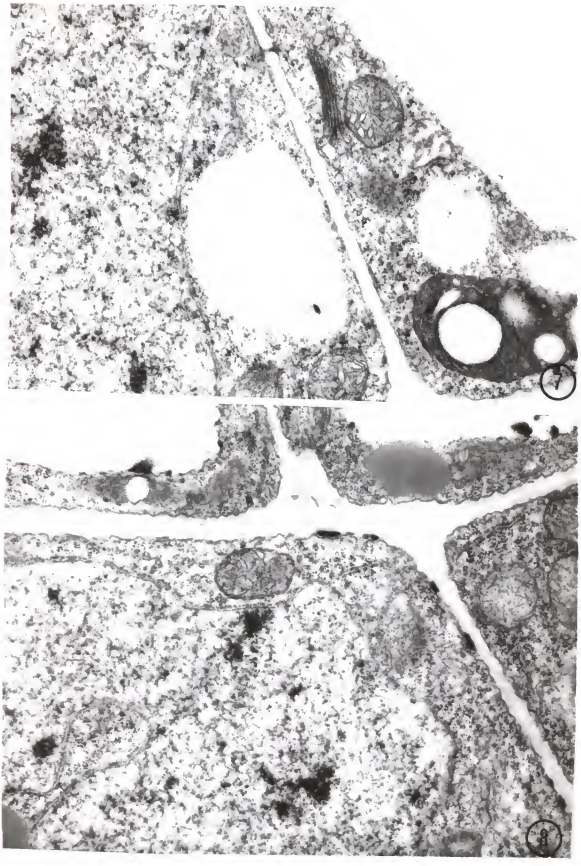
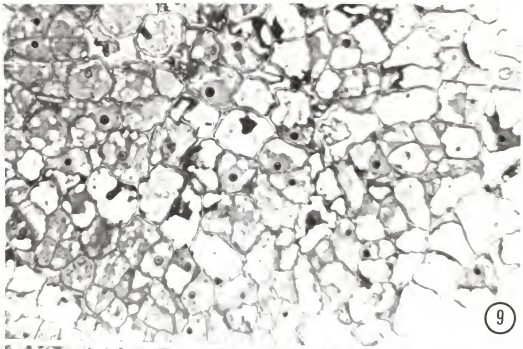


Fig. 9. Light micrograph of three day old cells grown in MS2C medium supplemented with 6% mannitol. Small cells with dense cytoplasm are seen along the periphery and highly vacuolated larger cells are found at the center. The multivacuolar nature of the cells is also apparent. x 280

Fig. 10. Electron micrograph of a cell pregrown in the presence of mannitol showing the presence of many small vacuoles throughout the cytoplasm. Nucleus, plastids, mitochondria, ER and golgi are also seen in the cytoplasm. x 10K



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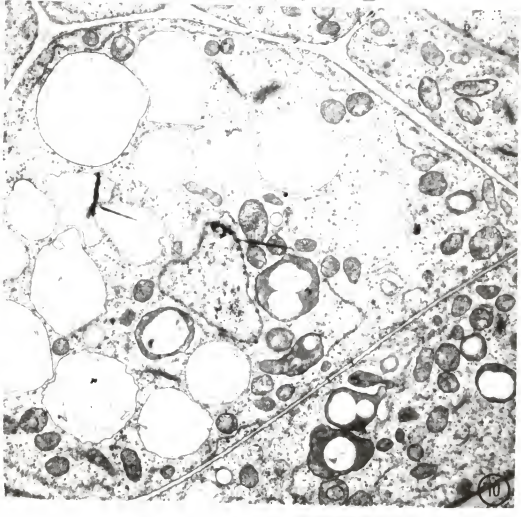
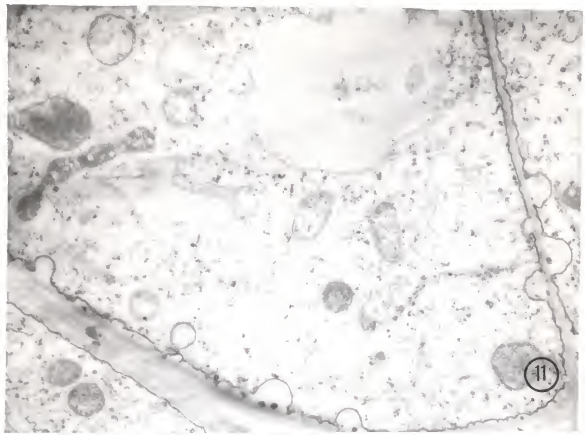


10

Figs. 11 and 12. Plasma membrane of cells grown in high osmoticum showing the presence of invaginations.

11. x 16K

12. x 16K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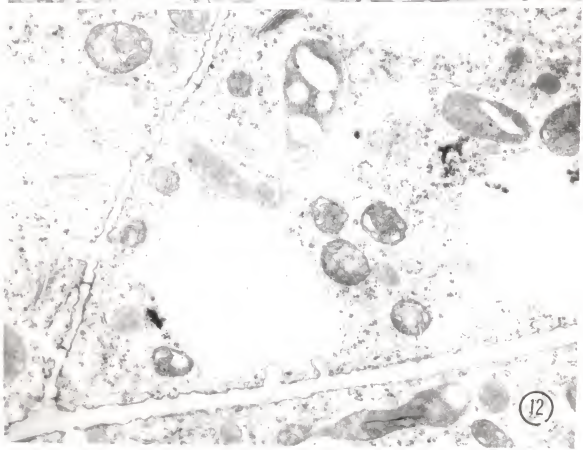
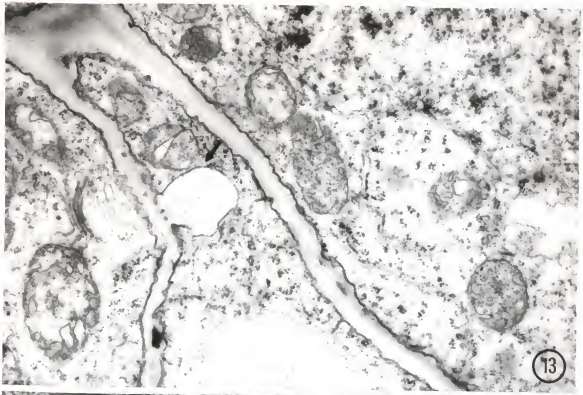

12

Figs. 13 and 14. Presence of invaginations in cells grown in a medium supplemented with mannitol.

13. x 26K

14. x 26K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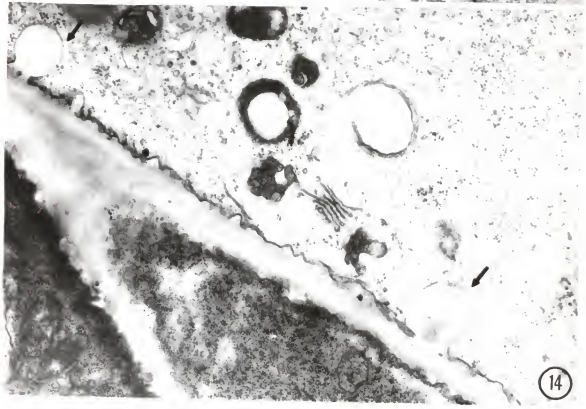

14

found to be 13.86 $\mu m^2/\mu m^3$, whereas in mannitol pregrown cells it was 18 $\mu m^2/\mu m^3$. The volume of the ER in relation to the control was 124%.

After treatment with the cryoprotectants the dilation of organelles was observed to be more pronounced. The average volume of the mitochondria was found to be 161% of those found in the control cells. ER was more prominent and could be clearly seen (Figs. 15, 16). The volume occupied by the ER in the cell was 1.7%. The area of the ER was found to be 26.6 $\mu m^2/\mu m^3$. The volume of the ER was 186% of the control tissue. Multimembranous structures were also observed in the cytoplasm (Fig. 17). The tonoplast also formed invaginations (Fig. 18), which eventually formed vesicles found inside the vacuole (Fig. 19).

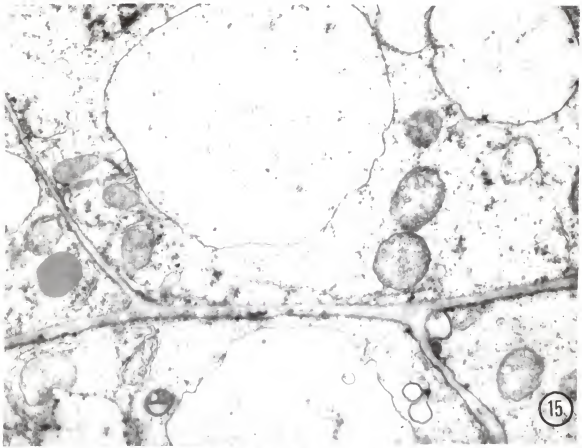
Survival of cells after cryoprotectant treatment was determined by TTC (2,3,5-triphenyl tetrazolium chloride) assay as well by regrowth potential (See chapter 4), to determine whether any viability loss has occurred due to treatment with the cryoprotectants. The percent survival obtained was 98% according to TTC test and 83% according to regrowth potential.

Membranous structures were found to be intact in cells subjected to pregrowth and cryoprotectant treatments and freeze-fixed at -10°C (Figs. 20, 21). Mitochondria and membrane bound nucleus could also be observed (Fig. 22). Again the mitochondria and ER were dilated, the volume of a mitochondrion being 265% of those from the control tissue. The volume of the ER was 1.3% of the total volume of the cell and 172% when compared to that of the control. The area was 21 $\mu m^2/\mu m^3$. The ER cisternae dilated and appeared vesicular and could be distinguished by the presence of ribosomes along the membrane. Damage to the plasma membrane and leakage of cell content were observed in some cells. Osmiophilic granules were observed along the plasma membrane in some cells that were lethally damaged during the freezing process (Fig. 23).

Figs. 15 and 16. Cells after treatment with the cryoprotectants.

15. x 16K

16. 16K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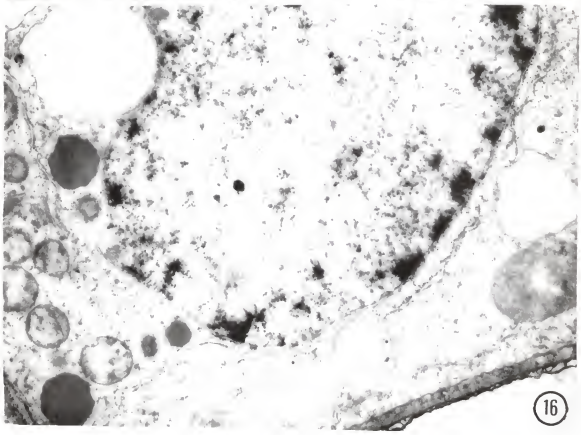
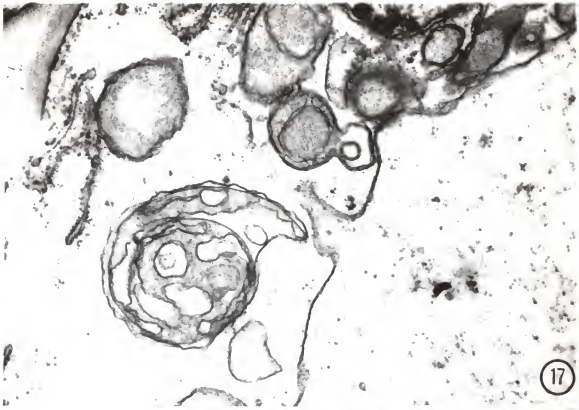

16

Fig. 17. Cell after treatment with cryoprotectants showing formation of multi-membranous structures. x 25K

Fig. 18. An invagination found in the tonoplast (arrow) after treatment with cryoprotectants. Nuclear envelope, ER and mitochondria are also seen. x 32K



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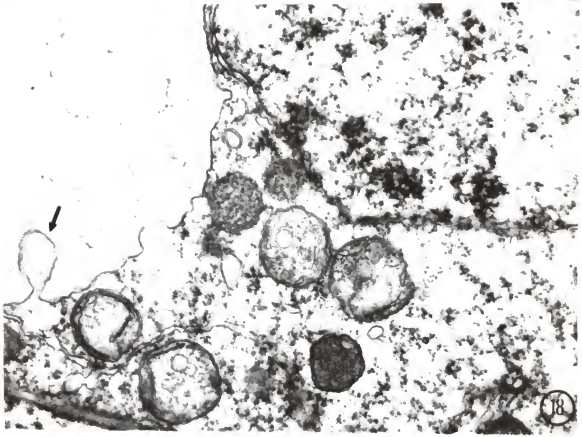
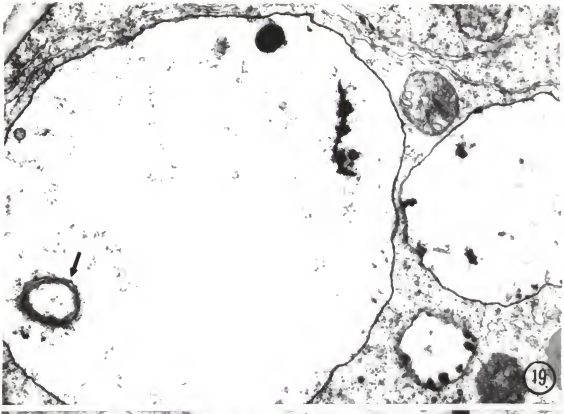


18

Fig. 19. A membrane bound vesicle is found inside the vacuole (arrow) of a cell after treatment with cryoprotectants. x 26K

Fig. 20. Cells pregrown in a medium containing mannitol and cryopreserved with 0.5 M sorbitol and 5% DMSO, and freeze-fixed at -10°C after cooling at a rate of 0.5°C/min. Numerous mitochondria, nucleus, and ER can be distinguished. x 13.2K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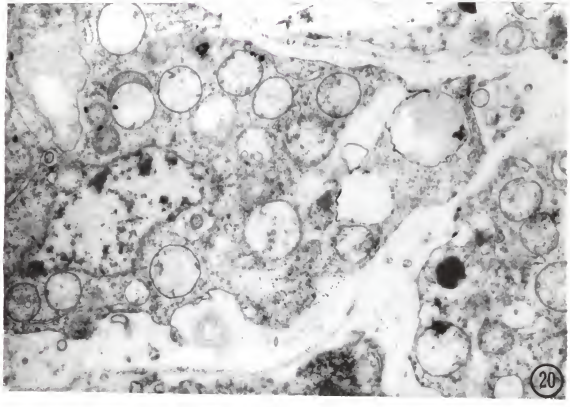
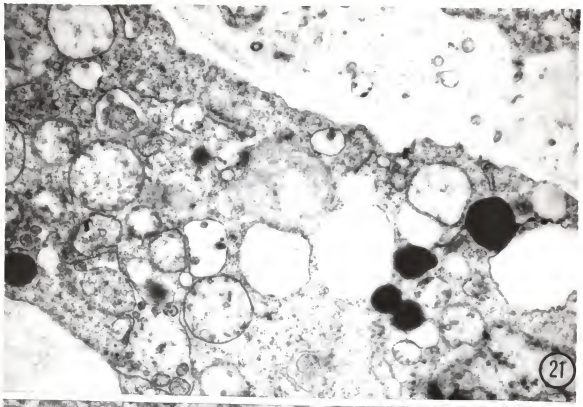

20

Fig. 21. A cell freeze-fixed at -10°C after treatment with the cryoprotectants. Dilated mitochondria and ER cisternae are seen x 16.6K

Fig. 22. Same as Fig. 21. Membrane bound nucleus, mitochondria and ER are seen. x 16.6K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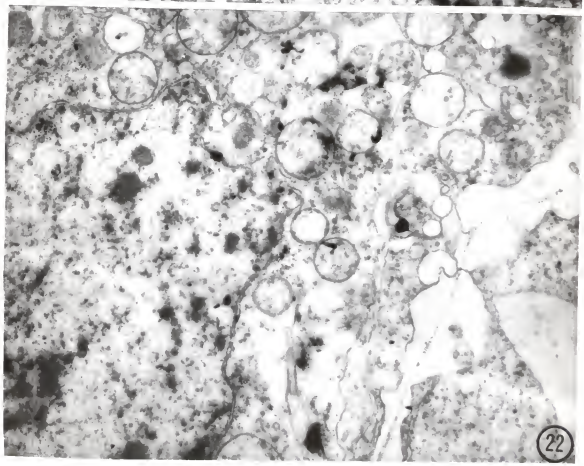
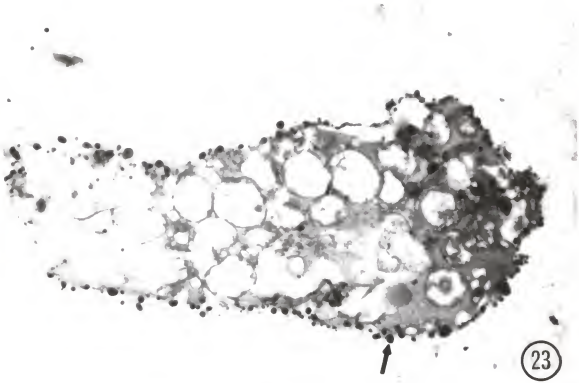

22

Fig. 23. A cell freeze-fixed at -10°C after treatment with cryoprotectants. The cell is lethally damaged, and osmiophilic granules are observed along the periphery of the plasma membrane. x 13.2K

Fig. 24. A cell freeze-fixed after slow freezing without any cryoprotection. The cell is plasmolyzed. Nucleus with a nucleolus is present. x 10K



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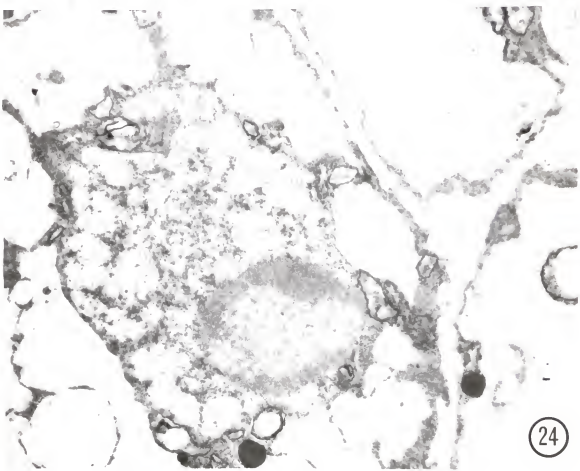


24

When cells were freeze-fixed without any treatments prior to freezing, the cells were lethally damaged. Very few, if any, intact organelles or membranous structures were observed. The cells also exhibited signs of plasmolysis (Fig. 24). Disruption of the membrane and plasmodesmatal connections could also be seen (Fig. 25).

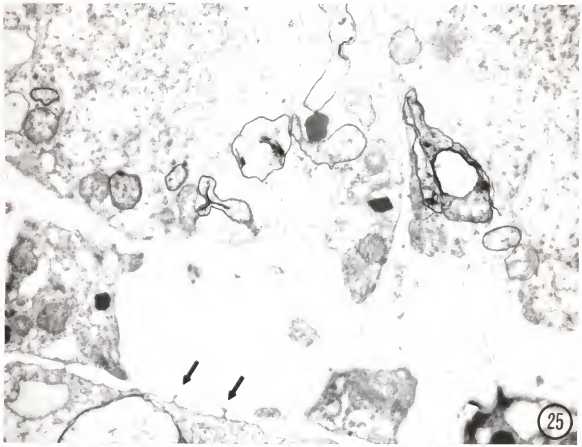
When cells were observed immediately after thawing, organelles and membranous structures could still be seen (Figs. 27-31). Nuclear membrane was intact (Fig. 28), and mitochondria, chloroplast and ER could be clearly distinguished. The average volume of the mitochondria was 217% of those in the control tissue. ER cisternae dilated extensively and formed vesicles and occupied 3% of the total volume of the cell. The area of the ER was 18.2 $\mu m^2/\mu m^3$. The volume of the ER was 336% of the control. Osmiophilic granules were noticed along the membrane in very few cells (Figs. 30-33). They were normally found on the outer surface of the membrane facing the cell wall (Figs. 30, 32), which could be clearly seen in cells where the plasma membrane was pulled away from the wall (Fig. 33). When the viability of cells was tested immediately after thawing, it was found to be 99% according to TTC test and 103% according to regrowth potential. The cells resumed growth after a lag period of two days, and no browning was observed (See chapter 4).

In cells fixed two days after thawing, the organelles were no longer dilated. The plasma membranes were smooth (Figs. 34, 35), and osmiophilic granules were absent. After ten days the cells looked similar to the control cells except for the absence of a central vacuole (Fig. 36).

Cells frozen rapidly by direct immersion into liquid nitrogen without any cryoprotective treatment were lethally damaged and when thawed lacked most membranous structures (Fig. 37).

Fig. 25. Cells freeze-fixed at -10°C without any cryoprotection. Disruption to the plasma membrane and plasmodesmatal connections can be seen. x 13.2K

Fig. 26. Cells immediately after thawing showing the presence of intact nucleus. Mitochondria and ER cisternae are also seen. x 10K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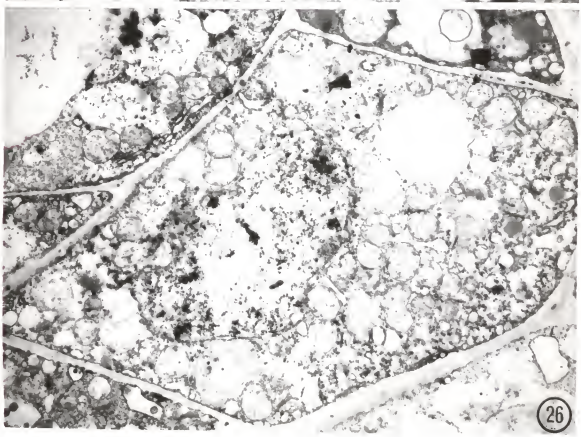
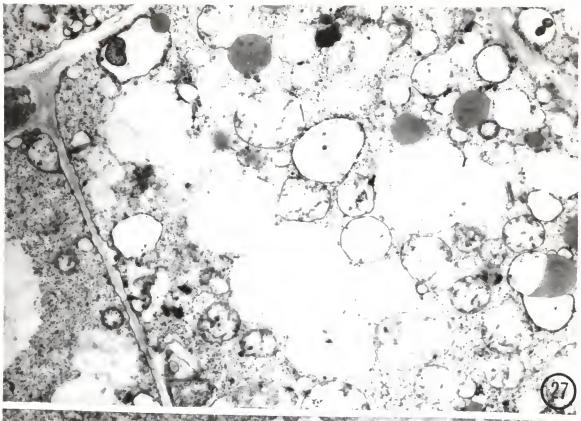

26

Figs. 27 and 28. Contents of cells immediately after thawing. The cells were cryopreserved using the optimized procedure.

27. x 16K

28. x 16K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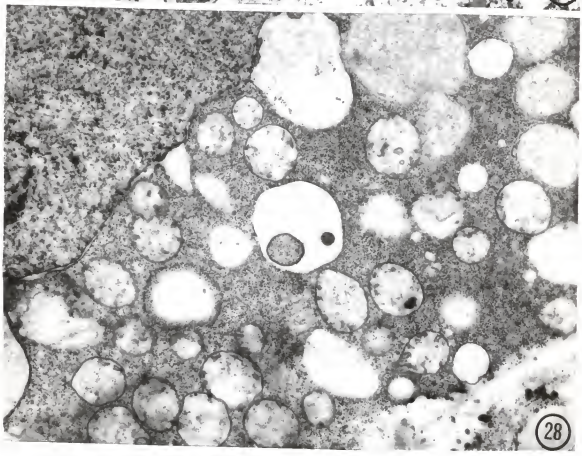
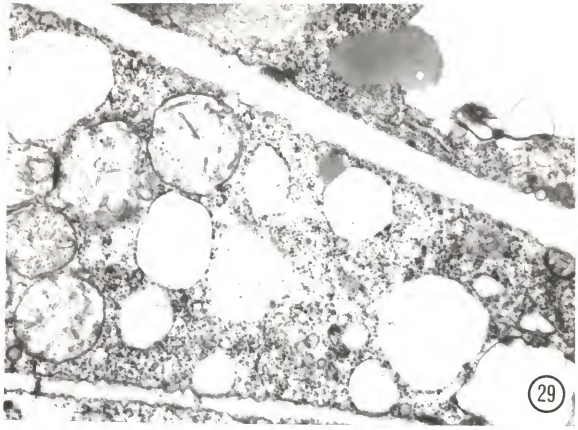

28

Fig. 29. A cell immediately after thawing. Plasma membrane with plasmodesmatal connections is seen. Mitochondria and dilated ER are also seen. x 26K

Fig. 30. Osmiophilic granules (arrow) are seen along the plasma membrane in a cell immediately after thawing. Plastids and mitochondria can also be seen. x 26K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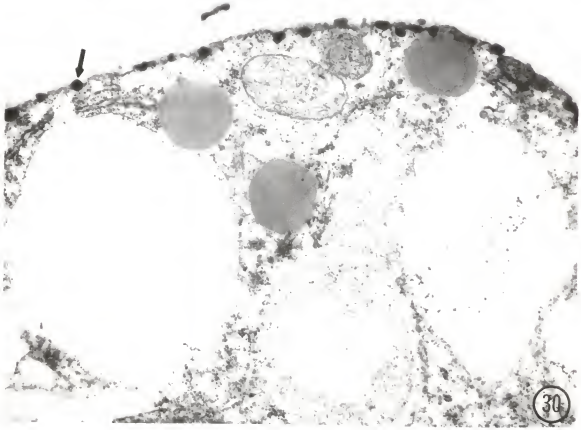

30

Fig. 31. A Cell fixed immediately after thawing showing the presence of dilated ER and mitochondria.x 16K

Fig. 32. Presence of osmiophilic granules (arrow) along the surface of the membrane in a cell immediately after thawing. x 26K

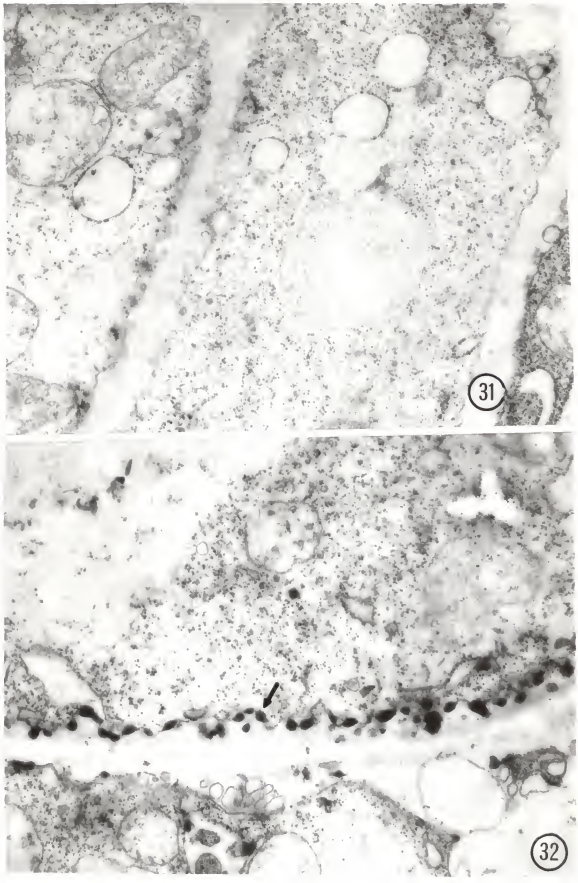
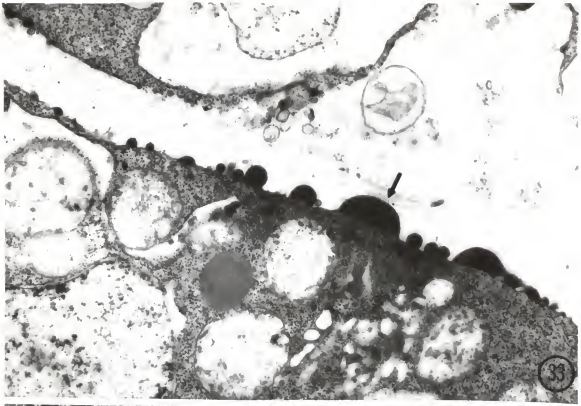

31
32

Fig. 33. Osmiophilic granules are clearly seen on the outer surface of the membrane. The plasma membrane is pulled away from the cell wall. x 26K

Figs. 34. Cells fixed two days after thawing showing membrane bound nuclei, nucleoli, plastids, ER, mitochondria, golgi and vacuoles. x 10K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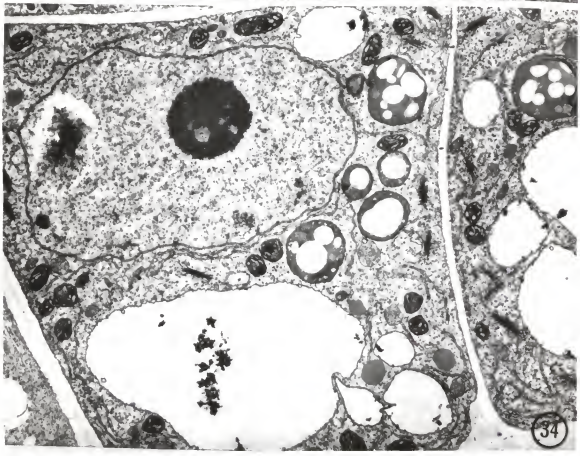
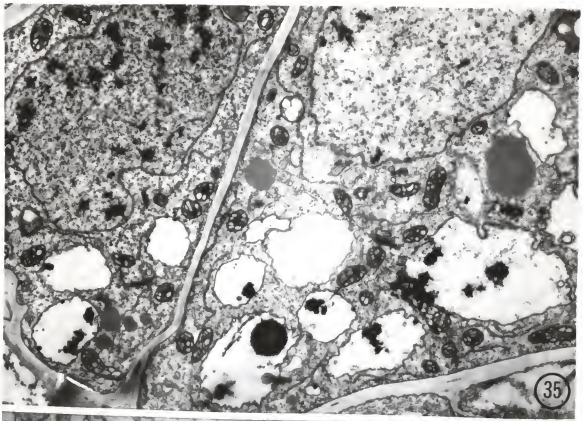

34

Fig. 35. Cells fixed two days after thawing showing nuclei, nucleoli, mitochondria and ER. x 10K

Fig. 36. Cells fixed ten days after thawing showing nuclei, nucleoli, mitochondria and ER. x 10K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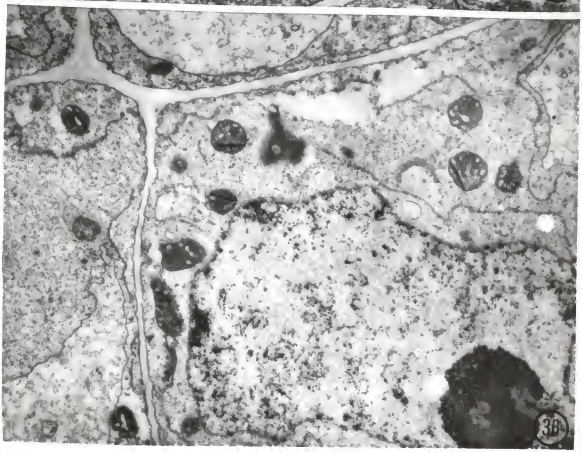
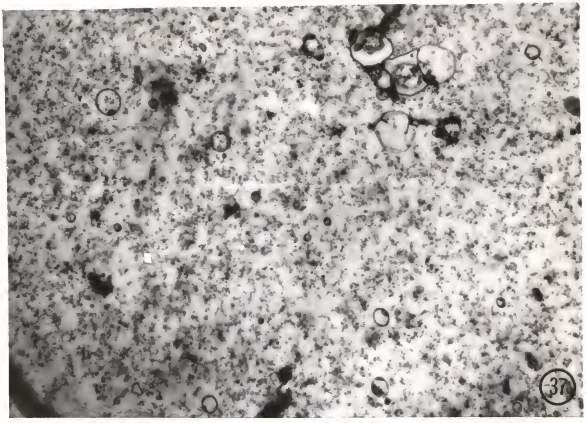

36

Fig. 37. Cells frozen rapidly without any cryoprotective treatments. Cells is lethally damaged and tiny vesicles are the only membranous structures present. x 13.2K

Fig. 38. Same as Fig. 37. Damage to the plasma membrane (arrow) can be seen. Some membranous structures are seen in the cytoplasm. x 48K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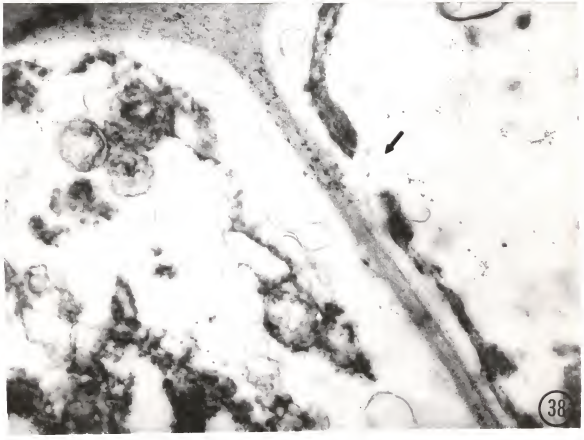
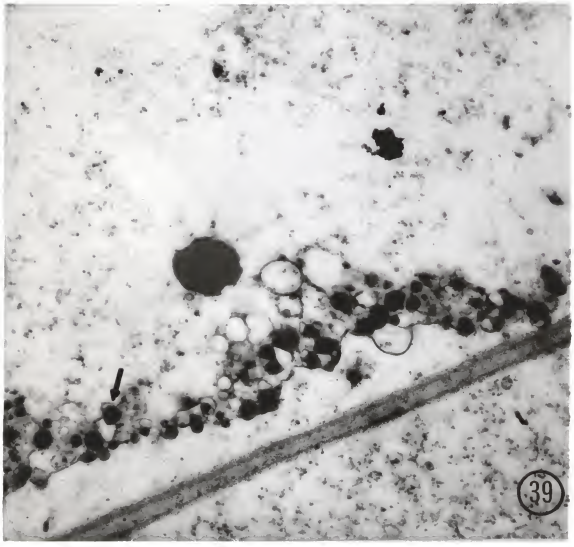

38

Fig. 39. Presence of osmiophilic granules along the plasma membrane in a cell frozen without any cryoprotection. x 25K


39

Plasma membrane was not clearly visible, and even in cells where it could be observed damage to the plasma membrane was clear (Fig. 38). Osmiophilic granules were seen along the plasma membrane in some cells (Fig. 39). When plated on culture medium these cells did not recover.

Discussion

Cell injury can manifest in a number of ways during freezing of biological material, and alterations in the membrane properties play a major role in causing this injury (Steponkus 1985a,b). *Panicum maximum* cells, when frozen slowly or rapidly without any pregrowth or cryoprotective treatments did not survive the freezing process. When pregrown in 6% mannitol, cryoprotected with 0.5 M sorbitol and 5% DMSO and cooled slowly at a rate of 5°C/min, marked improvement in survival was obtained, and more than 90% of the cells resumed growth when thawed and plated (See chapter 4).

Changes were observed in the ultrastructure of the *P. maximum* cells during different stages of the cryopreservation procedure. When pregrown in mannitol, a reduction of 11% was observed in the volume of the cells. The control cells were larger with a central vacuole, whereas the cells in mannitol either lacked a vacuole or were multivacuolar in nature. A considerable reduction in vacuolar volume was also observed. The vacuole occupied only 23% of the volume of the cell instead of the 58% observed in the control cells. Reduction in the vacuolar volume has also been reported in sycamore (*Acer pseudoplatanus*) cells grown in the presence of mannitol (Pritchard *et al.* 1982). A *Catharanthus* cell line with high freeze tolerance was also reported to be of multivacuolar type (Kartha *et al.* 1982).

Cell injury can be either mechanical from the formation of intracellular ice or by "solution effects" arising from the concentration of solutes in the cells due to the formation of extracellular ice (Mazur *et al.* 1972). Protective dehydration is essential for prevention of freeze injury, whereby the cell water content is reduced to a level where excess water is not present to cause structural damage by formation of intracellular ice crystals, but not severe enough to cause damage by "solution effects."

Mannitol acts as an osmoticum causing removal of water from the cell, hence exerting protective dehydration. By redistribution and reduction of the vacuolar volume the amount of water is reduced. Major changes were also observed in the plasma membrane of the cells grown in mannitol. Invaginations were observed from the plasma membrane into the cytoplasm. These probably form to accommodate the volume reduction caused during growth in mannitol containing medium.

Protoplasts isolated from acclimated and non-acclimated plants of rye (*Secale cereale* L. cv. Puma) behaved differently when observed using a cryomicroscope during cooling to sub-zero temperatures (Dowgert and Steponkus 1984). Temperature of nucleation was found to be reduced in acclimated tissue (Steponkus *et al.* 1982a; Dowgert and Steponkus 1983). During osmotic contraction protoplast from non acclimated plants produced endocytotic vesicles which were found in the cytoplasm, but during osmotic expansion the vesicles remained in the cytoplasm and the cell lysed before it reached its original volume, a process referred to as expansion induced lysis. Protoplasts from acclimated tissue produced exocytotic extrusions, which were drawn back into the surface of the protoplast during osmotic expansion and the cell regained its original volume (Dowgert and Steponkus 1984). Loss of osmotic responsiveness was the predominant form of injury in acclimated tissues.

Protoplasts of non-acclimated and acclimated tissue, when subjected to hypertonic solution contracted osmotically, and when transferred back to an isotonic solution they expanded, a process similar to cooling and rewarming. Therefore micro-osmotic manipulation was also used to study the mechanisms involved in cryopreservation (Wiest and Steponkus 1977a,b, 1978; Gordon-Kamm and Steponkus 1984a,b; Dowgert *et al.* 1987).

Using thin sections and fluorescein-Con-A labelling techniques along with micro-osmotic manipulation it was confirmed that the vesicles found in protoplasts of rye during osmotic contraction were derived from the plasma membrane (Gordon-Kamm and Steponkus 1984a), and that exocytotic extrusions were also bound by the plasma membrane with an osmiophilic interior which was composed of lipid material preferentially lost from the plasma membrane during osmotic contraction (Gordon-Kamm and Steponkus 1984b). They concluded that with non-acclimated protoplasts entire regions of the plasma membrane were pinched off during osmotic contraction and numerous vesicles were liberated into the cytoplasm, but during osmotic expansion the vesicles were not readily reincorporated into the membrane, hence causing lysis of the cell (Gordon-Kamm and Steponkus 1984a). In protoplasts of acclimated tissue the exocytotic extrusions never completely separated from the membrane and were drawn back into the plasma membrane during osmotic expansion, thereby preventing lysis of the cell (Gordon-Kamm and Steponkus 1984b).

The stresses experienced by protoplasts during osmotic contraction and expansion may be different in the presence and absence of the cell wall. After osmotic manipulation of intact cells of cold hardened and non-hardened winter rye, Singh (1979b) reported the occurrence of osmiophilic granules in cells of both hardened and non-hardened tissue, but it occurred at a higher osmotic stress in hardened cells. When these tissues were frozen to -10°C, presence of osmiophilic

granules was observed in the non-hardened cells, whereas they were absent from the hardened tissue, where 100% survival was obtained after exposure to this temperature. In non-hardened cells, the cellular membrane rolled up forming multibilayered vesicles, which eventually lost their lamellar lattice forming osmiophilic bodies, whereas invaginations were observed in the plasma membrane of hardened tissue (Singh and Miller 1985). According to Singh (1979b), osmiophilic bodies were formed by the irreversible loss of membrane material, which caused the cells to lyse during reexpansion.

As the cells are plasmolyzed by protective dehydration, the area of the membrane is not conserved (Steponkus and Wiest 1978, 1979; Wiest and Steponkus 1978; Gordon-Kamm and Steponkus 1984a). Cells underwent plasmolysis when pregrown in mannitol, and the plasma membrane probably formed invaginations to accommodate the reduction in the cell volume. The evidence is not enough to determine whether they eventually form vesicles that are liberated into the cytoplasm.

The cells frozen by the optimized procedure for cryopreservation of *P. maximum* cell suspension (See chapter 4), retained most of the membranous structures and the organelles. Nuclear membrane, organelles and plasmodesmatal connections were seen in cells immediately after thawing. Dilation of the mitochondria and ER occurred throughout the cryopreservation procedure, the ER cisternae forming vesicles due to the dilation. Dilation of the organelles has also been reported in carrot and sycamore cells during the process of cryopreservation (Withers 1978b; Withers and Davey 1978). Some damage to the membranous structures as well as leakage of cytoplasmic contents was observed in some cells. When plated on semi-solid culture medium the cells resumed growth after a lag period of two days and grew at rates comparable to the control. Therefore, the damage incurred during the cryopreservation procedure probably gets repaired

within a very short period of time. This was confirmed by fixing cryopreserved and thawed cells after two days in culture. The cells were found to be similar to the control cells without any apparent damage to the ultrastructure.

The presence of osmiophilic granules in lethally damaged cells indicated that they could not have formed as an adaptation to prevent freeze damage. Although they are formed along the membrane, at this point their origin is not determined.

Cells freeze-fixed at -10°C without any cryoprotection showed signs of plasmolysis. The plasma membrane was starting to pull away from the cell wall, and in some cells the disruption of the plasma membrane and plasmodesmatal connections could be observed (Fig. 25). When cells were frozen without any cryoprotection, protective dehydration could not have taken place. This would have resulted in the formation of intracellular ice crystals during freezing, and the consequent structural damage.

When cells were frozen rapidly by direct immersion into liquid nitrogen without any cryoprotective treatment, all the membranous structures including the plasma membrane were extensively damaged. When cells were frozen without any cryoprotective treatments protective dehydration did not take place. In addition when frozen at rapid rates, there is not enough time for the water to leave the cell, and intracellular ice crystallization takes place. These may not damage the cells in the frozen state but may injure the cells during the rewarming process (Farrant *et al.* 1977). Suspension cultures of Sycamore (*Acer pseudoplatanus* L.), when frozen rapidly or slowly without treatment with cryoprotectants suffered lethal damage by intracellular ice formation or cellular dehydration (Withers and Davey 1978). Osmiophilic granules were observed in lethally damaged cells of *Panicum maximum* further indicating that it is not an adaptation to prevent freeze damage in cells. Their presence was not observed in all the cells, and at this point it is not known whether they are reincorporated into the plasma membrane.

Therefore it can be inferred that protective dehydration, and cryoprotectant treatment are very important for the cells to survive sub-zero temperatures. The extend of damage to the cell varies depending on the treatment employed. When frozen by the optimized procedure, although some damage was apparent the membranous structures were still intact. The cells could repair the damages in a short period of time and resume growth. The cells frozen without any cryoprotection were extensively damaged. This damage could not be repaired and the cells did not recover when plated on semi-solid medium.

CHAPTER 4

OPTIMIZATION OF THE CRYOPRESERVATION PROCEDURE FOR STORAGE OF CELL SUSPENSIONS

Introduction

Cell suspension cultures are used extensively in secondary product synthesis, studies of physiology and morphology, isolation of mutant cell lines, protoplast isolation and genetic engineering (Vasil and Vasil 1980; Withers 1983; Hauptmann *et al.* 1987; Vasil 1987, 1988; Vasil *et al.* 1988, 1990). Their maintenance is a labor intensive process which could result in the loss of cell lines, in addition to loss of morphogenetic potential and accumulation of mutations.

It will be very useful, therefore, if cell suspensions can be stored at stable conditions for extended periods of time, eliminating the requirements for periodic subculture. Cryopreservation has been extremely successful for storage of a number of cell suspension cultures. It involves a number of steps, each of which is important to attain high survival rates. *Panicum maximum* and *Pennisetum americanum* cell suspensions were used in this study to optimize the cryopreservation procedure at each stage involved.

Materials and Methods

Cell suspensions of *Panicum maximum* (established in 1985 from leaf tissues as described in Lu and Vasil 1981a,b), and *Pennisetum americanum* (established in 1986 from immature embryos as described in Vasil and Vasil 1981) have been

routinely maintained by transferring 12 ml of the cell suspension into 35 ml of fresh MS2C medium in 250 ml Erlenmeyer flasks at weekly intervals. Both these cell lines were used to study and optimize each step of the cryopreservation procedure.

Effect of pregrowth treatments and cryoprotectants on survival

Cells were pregrown in MS2C alone, or supplemented with either 6% (w/v) mannitol or 6% (w/v) sorbitol prior to cryopreservation. Twelve ml of the seven day old culture was transferred to 35 ml of the pregrowth medium, and used for cryopreservation experiments after three days in culture.

Two different combinations of cryoprotectants consisting of 0.5 M DMSO + 0.5 M glycerol + 1 M sucrose or 0.5 M sorbitol + 5% DMSO were used, in combination with each of the pregrowth treatments. Cryoprotectants were prepared in double strength, filter sterilized and chilled on ice. The compounds were dissolved in water except in one experiment where they were dissolved in MS2C medium.

Within a single experiment, the same packed cell volume (PCV) of cells was used to avoid differences in response that may arise due to the differences in cell densities. Fifteen ml of the suspension was dispensed into sterile centrifuge tubes and spun at 100 x g for 3 minutes. The PCV was then adjusted to the required value. It was taken into account that the final PCV obtained was half of the initial value, due to the addition of an equal volume of cryoprotectant solution. A final PCV of 20 % was used in most experiments unless stated otherwise. The suspension was then transferred to a 125 ml Erlenmeyer flask and chilled on ice. Prechilled cryoprotectant prepared in double strength was added to the cell suspension in ten increments over a one hour period, while the suspension was still being maintained on ice on a rotary shaker. After addition the cells were left for one more hour on the shaker to facilitate the uptake of cryoprotectants.

One ml of the cell suspension was dispensed into 1.2 ml polypropylene, screw cap ampoules and cooled in a Cryomed Model 1010 Micro Computer Programmable Freezer Unit at a rate of 0.5°C/min. Temperature of the sample was monitored by inserting a thermocouple into one of the ampoules. The ampoules were cooled to a transfer temperature of -40°C, and held at that temperature for 40 minutes before transfer to liquid nitrogen. The ampoules were stored for at least two weeks in liquid nitrogen, before thawing to determine viability. Thawing was carried out rapidly by plunging the ampoules into a water bath at 40°C with stirring. The ampoules were removed when the ice had just disappeared, and wiped with 95% ethyl alcohol before opening.

Viabilities were determined by TTC reduction assay as well as by regrowth potential. For TTC reduction assay the contents of the ampoules were transferred to 15 ml centrifuge tubes and 3 ml of the TTC solution was added and left in total darkness for 16 hours. The tubes were then centrifuged and the supernatant was removed. The cells were rinsed with double distilled water and the supernatant was again removed after centrifugation. Seven ml of 95% ethyl alcohol was added next, and the tubes with the contents were heated for 5 minutes in a water bath at 80°C. After cooling, the volume of each tube was adjusted to 10 ml using 95% ethyl alcohol. Absorption was measured at 530 nm using a Beckman DU-40 Spectrophotometer and the viabilities were determined by comparing the values obtained from the cryopreserved cells with those of the control cells.

Viabilities were also determined by regrowth potential, which was based on the increase in the fresh weight of the cryopreserved cells during culture. Filter papers sterilized by autoclaving were placed on Petri dishes containing 25 ml of MS2C medium solidified with 0.2% gelrite. The weight of the filter papers was determined once they were saturated with water. The thawed ampoules were opened after wiping with alcohol, and contents of each ampoule were poured onto

the filter paper layered on MS2C medium. The filter paper along with the cells was weighed again after 30 minutes and every two days thereafter. Fresh weight of the cells was calculated by deducting the weight of the filter paper alone, from the weight of the filter paper with the cells growing on it. Growth curves were drawn by plotting the fresh weight of the tissue against time. The combination of pregrowth and cryoprotectants that gave the highest viability was used hereafter in all experiments to further optimize viability.

Rate of cooling

For optimization of cooling rates a final PCV of 20% was used. Cells were prepared for freezing as described before, and cooled at rates of 5°C/min, 1°C/min and 0.5°C/min to -40°C and held at that temperature for 40 minutes before plunging into liquid nitrogen. After storage they were thawed rapidly and the viabilities were determined by TTC reduction assay. The rate of cooling which resulted in the highest survival was used hereafter in all experiments.

Transfer temperature

Cells at a final PCV of 20% were cooled at a constant rate of 0.5°C/min to different transfer temperatures and plunged immediately into liquid nitrogen without a holding period. The transfer temperatures employed were -20°C, -40°C and -60°C. Survival was determined by TTC reduction assay once the cells were thawed rapidly after two weeks in liquid nitrogen.

Packed cell volume and holding time

Cells with final packed cell volumes of 10%, 20% and 40% were cryopreserved by the same procedure, using a cooling rate of 0.5°C/min. For all three experiments the same transfer temperature of -40°C was used, but the holding time at this temperature varied. Some ampoules were transferred immediately into liquid nitrogen once they reached -40°C, some were held at this temperature for 40 minutes as in all previous experiments and the rest were held for a total of 80 minutes. The cells were thawed rapidly and the viabilities were determined by TTC reduction assay.

Cryoprotectants dissolved in water vs. in culture medium

Sorbitol (0.5 M) and DMSO (5%) were dissolved in water as well as in liquid MS2C medium to determine effect on survival. PCV of 20% was used and the cells were frozen at 0.5°C/min to -40°C and plunged immediately into liquid nitrogen without a holding period. Viabilities were determined by TTC reduction assay after rapid thawing.

Uptake time

In all previous experiments, after the addition of cryoprotectants cells were left for an hour to enhance the uptake of cryoprotectants before freezing. To determine whether prolonged exposure has any deleterious effects on survival, cells were frozen immediately after the completion of addition of cryoprotectants as well as after a one hour uptake period. PCV of 20% was used, and cells were frozen at 0.5°C/min to -40°C and plunged immediately into liquid nitrogen. Survival was determined by TTC reduction assay after thawing.

The cryopreservation procedure was optimized from the results obtained from all the experiments described above. The cells were again cryopreserved by combining all the factors that gave the highest viability in each stage. Cells were pregrown in mannitol and the PCV was adjusted to 80% so that a final PCV of 40% could be obtained. An equal volume of double strength cryoprotectant (0.5 M sorbitol and 5% DMSO) prepared in water, was added next over a one hour period, and the suspension was transferred to ampoules without an uptake period. The cells were cooled at a rate of 0.5°C/min to -40°C and immediately transferred to liquid nitrogen without any holding period.

Cells were thawed rapidly by plunging the ampoules into a water bath at 40°C, and slowly by allowing the ampoules to thaw in air at room temperature (about 15 min). Survival was determined by TTC test and regrowth potential at different stages of the cryopreservation procedure, such as, after treatment with cryoprotectants, after cooling to -40°C, and after rapid and slow thawing to determine whether any viability loss occurred at any of these stages. Viability was also checked at different time intervals, over a period of three years to determine whether long term storage in liquid nitrogen caused progressive deterioration in the survival.

The procedure optimized from the results obtained was used for cryopreservation of various cell lines.

Protoplast isolation

Cell suspension of *Panicum maximum* was used for isolation of protoplasts from both control and cryopreserved cells. Cryopreserved cells were thawed and grown on semi-solid MS2C medium for a month, and then transferred into 35 ml of liquid MS2C medium in 250 ml Erlenmeyer flasks. The cryopreserved cell line was

maintained by transferring 12 ml of the cell suspension into 35 ml of fresh MS2C medium at weekly intervals.

Three day old control and cryopreserved cells were transferred into fresh culture medium and used for protoplast isolation on the second day. The PCV of both cell lines was adjusted to 40% to standardize the experiment.

Enzyme mixture was prepared by dissolving 1% Cellulase R S (Yakult Honsha), and 0.8% Pectinase (Serva) in MES buffer, and the pH was adjusted to 5.6 before filter sterilization. A small volume (1.5 ml) of each suspension was transferred to 15 ml of the enzyme mixture in Petri dishes and incubated at room temperature on a shaker for 1-2 hours, followed by overnight incubation at 12°C without shaking.

The protoplast-enzyme mixture was first filtered through a Miracloth, followed by 100 and 50 μm stainless steel filters. The mixture was then transferred to 15 ml centrifuge tubes and spun at 100 x g for three minutes. The supernatant was removed and the volume was brought to 10 ml with MES buffer and mixed well. A drop from the protoplast buffer mixture was used to determine the yield by hemacytometric counting. The protoplasts were washed two more times with MES buffer. The supernatant was removed after the last wash and centrifugation, and Kao and Michayluk's modified nutrient medium (Vasil and Vasil 1980) was added to the protoplasts. The volume of the culture medium was adjusted so that a final protoplast density of $1x10^5$/ml was achieved. The protoplasts in the culture medium were transferred to 35x10 mm Falcon Petri dishes and incubated in total darkness at 27°C. The plating efficiencies of protoplasts isolated from control and cryopreserved cells were determined after 10 days in culture. Plating efficiencies were calculated from the percentage of cultured protoplasts that formed colonies.

The optimized procedure was used for the cryopreservation of cell suspensions of *Pennisetum purpureum*, *Saccharum* hybrids (SCH and SH2). Percent survival was determined from regrowth potential on semi-solid culture medium.

Results

When cells of *Panicum maximum* and *Pennisetum americanum* were cryopreserved by using different pregrowth and cryoprotectant treatments, marked differences were observed in their viability (Tables 1, 2; Figs. 40, 41).

In *Panicum maximum* highest survival was obtained when the cells were pregrown in MS2C medium supplemented with either 6% mannitol or 6% sorbitol (Table 1). Rate of growth was determined for each treatment between days where the growth was found to be linear. The percent survival was calculated by comparing the rate of growth of the cryopreserved cells with that of the control. Highest survival was 71-75% based on TTC assay and 74-86% based on regrowth potential (Table 1; Fig. 40). Cells from Treatment 4 where a 28% survival was obtained based on TTC test, failed to resume growth in culture.

In *Pennisetum americanum* percent survival based on TTC test were considerably lower than those from regrowth potential (RP). A highest survival of 66% (TTC) and 91% (RP) was obtained when cells were pregrown in MS2C medium supplemented with 6% mannitol and cryoprotected with 0.5 M sorbitol and 5% DMSO, as with *Panicum maximum* cell line (Table 2; Fig. 41). When these cells were returned to culture a lag phase of about 6 days was observed, which may explain the lower results obtained from TTC test. The cells cryopreserved without any pregrowth treatments, especially those cryoprotected with 0.5 M sorbitol and 5% DMSO failed to survive when plated on semi-solid medium. The rate of growth

was determined from the difference in fresh weights between day 8 and day 18 during which time the growth was linear.

Cooling rate

The viabilities varied considerably when cells subjected to the same pregrowth and cryoprotectant treatments were cooled at different rates to the same terminal freezing temperature before transfer to liquid nitrogen (Table 3). A cooling rate of 0.5°C/min gave the highest viability with both cell lines, and the viabilities decreased with increased rates of cooling.

Terminal freezing temperature

The optimum terminal freezing temperature for both cell lines was found to be -40°C (Table 4). Survival was very low when cells were transferred to liquid nitrogen after a terminal freezing temperature of -20°C. Cooling beyond -40° also decreased viability.

PCV and holding time

Improved recovery of *Panicum maximum* and *Pennisetum americanum* cells was observed with increased PCV (Tables 5, 6). Final PCV of 40% gave the highest recovery. Within each PCV, recovery varied with the holding time, when all other parameters were kept constant. Highest survival was obtained when cells were transferred to liquid nitrogen, immediately after they reached -40°C, and decreased with increase in the holding time. This was found to be consistent within each PCV used.

Table 1: Survival of *Panicum maximum* cells, based on TTC test and regrowth potential (RP) subjected to different pregrowth and cryoprotectant treatments (averages of two experiments; there were five replicates for each treatment, with 1 ml of cells).

Treatment	Pregrowth medium	Cryoprotectants	% survival * TTC	RP
1	MS2C	0.5 M sorbitol 5% DMSO	31±1.86	52±4.66
2	MS2C + 6% mannitol	0.5 M sorbitol 5% DMSO	75±1.59	86±5.71
3	MS2C + 6% sorbitol	0.5 M sorbitol 5% DMSO	72±1.47	81±5.48
4	MS2C	0.5 M DMSO 0.5 M glycerol 1 M sucrose	28±0.50	00±0.00
5	MS2C + 6% mannitol	0.5 M DMSO 0.5 M glycerol 1 M sucrose	60±2.79	74±7.20
6	MS2C + 6% sorbitol	0.5 M DMSO 0.5 M glycerol 1 M sucrose	71±1.57	78±4.10

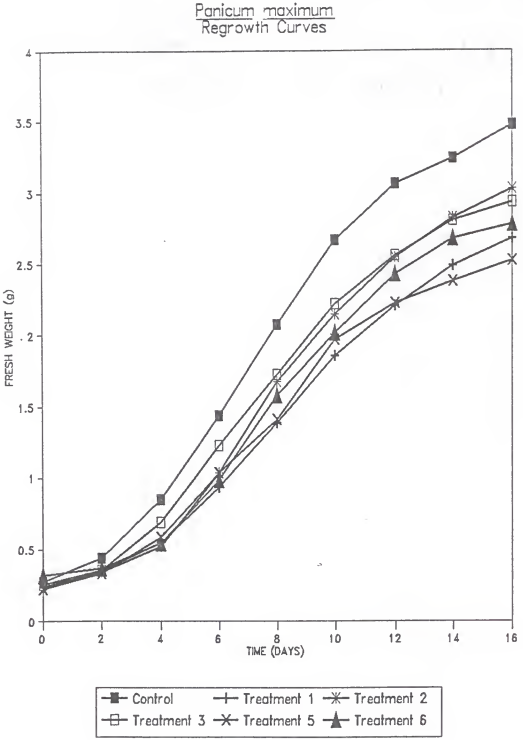
* Numbers represent the values of the mean ± standard error of the mean.

Table 2: Survival of *Pennisetum americanum* cells, based on TTC test and regrowth potential (RP) subjected to different pregrowth and cryoprotectant treatments (averages of two experiments; there were five replicates for each experiment, with 1 ml of cells).

Treat-ment	Pregrowth medium	Cryoprotectants	% survival * TTC	RP
1	MS2C	0.5 M sorbitol 5% DMSO	19±0.35	00±0.00
2	MS2C + 6% mannitol	0.5 M sorbitol 5% DMSO	66±1.62	91±3.48
3	MS2C + 6% sorbitol	0.5 M sorbitol 5% DMSO	56±1.33	78±1.44
4	MS2C	0.5 M DMSO 0.5 M glycerol 1 M sucrose	24±0.45	04±1.03
5	MS2C + 6% mannitol	0.5 M DMSO 0.5 M glycerol 1 M sucrose	42±0.99	69±6.20
6	MS2C + 6% sorbitol	0.5 M DMSO 0.5 M glycerol 1 M sucrose	30±2.00	19±4.14

* Numbers represent the values of the mean ± SEM

Fig. 40. Regrowth curves of control and cryopreserved cells of *Panicum maximum* cells plated on MS2C medium. Cells from Treatment 4 did not resume growth. (Averages of two experiments, five replicate plates for each treatment, with 1 ml cells/plate).


Panicum maximum
Regrowth Curves
FRESH WEIGHT (g)
TIME (DAYS)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
2
4
6
8
10
12
14
16
Control
Treatment 1
Treatment 2
Treatment 3
Treatment 5
Treatment 6

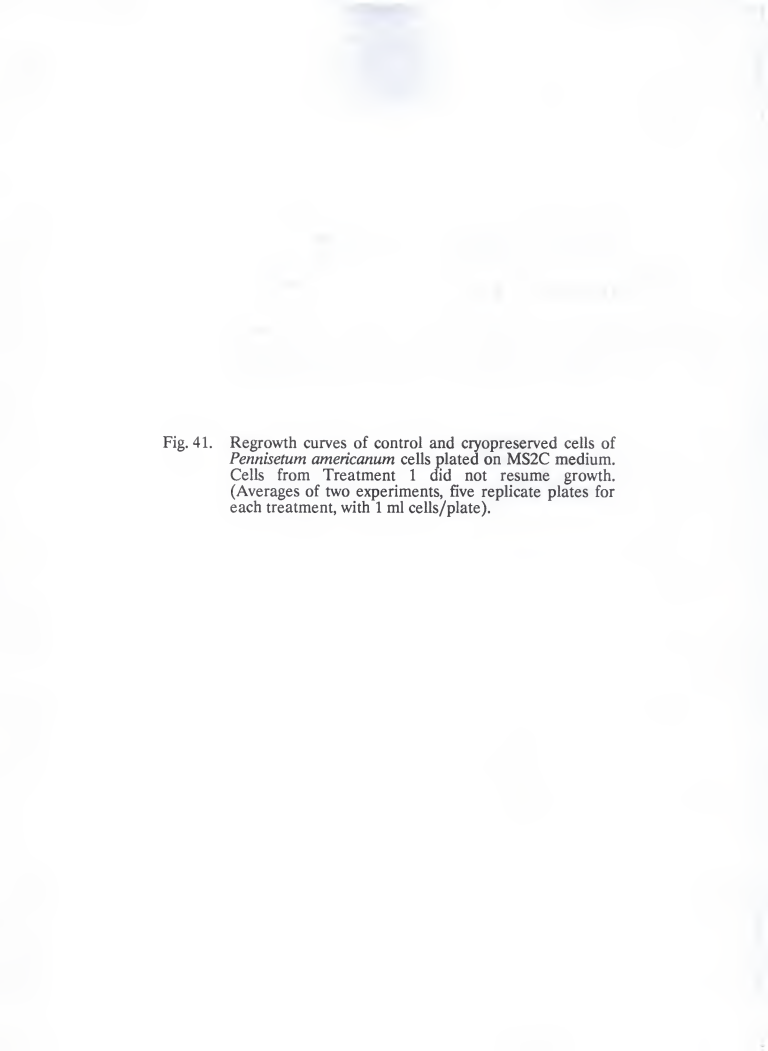


Fig. 41. Regrowth curves of control and cryopreserved cells of *Pennisetum americanum* cells plated on MS2C medium. Cells from Treatment 1 did not resume growth. (Averages of two experiments, five replicate plates for each treatment, with 1 ml cells/plate).

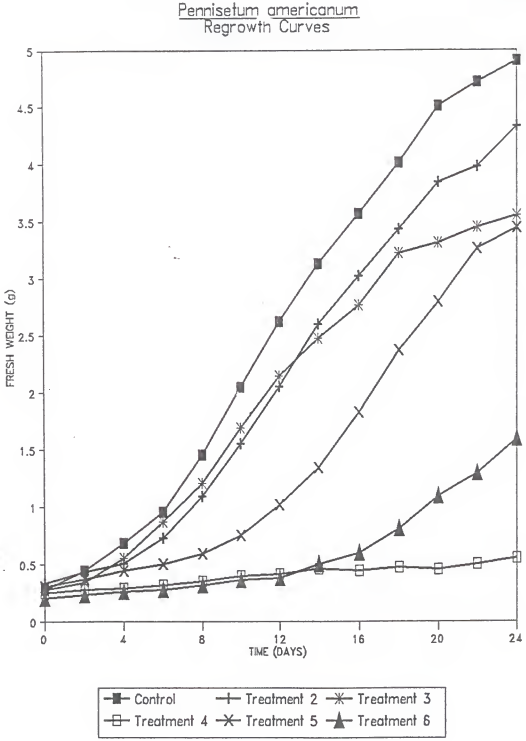

Pennisetum americanum
Regrowth Curves
FRESH WEIGHT (g)
TIME (DAYS)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
4
8
12
16
20
24
Control
Treatment 2
Treatment 3
Treatment 4
Treatment 5
Treatment 6

Table 3: Effect of cooling rate on survival of *P. maximum* and *P. americanum* cell suspensions, based on TTC reduction assay (averages of two experiments, five replicates per experiment).

Cooling Rate	% survival*	
	P. maximum	*P. americanum*
5°C/min	25±0.16	14±0.75
1°C/min	57±1.66	37±1.34
0.5°C/min	78±2.10	58±1.36

* Numbers represent the values of the mean ± SEM

Table 4: Effect of transfer temperature on survival of *P. maximum* and *P. americanum* cell suspensions based on TTC reduction assay (averages of two experiments, five replicates per experiment).

Transfer Temperature	% survival*	
	P. maximum	*P. americanum*
-20°C	23±0.96	10±0.23
-40°C	76±2.43	59±1.36
-60°C	55±1.61	33±0.84

* Numbers represent the values of the mean ± SEM

Table 5: Effect of PCV and holding time on survival of *P. maximum* cells. Survival is based on TTC reduction assay (averages of two experiments, five replicates per experiment).

		Final PCV		
		10%*	20%*	40%*
	0	66±2.41	85±1.64	88±1.69
Holding time	40	47±1.58	70±1.60	70±1.99
(min)	80	48±1.43	52±1.21	61±0.68

* Numbers represent the values of the mean ± SEM

Table 6: Effect of PCV and holding time on survival of *P. americanum* cells. Survival is based on TTC reduction assay (averages of two experiments, five replicates per experiment).

		Final PCV		
		10%*	20%*	40%*
	0	35±1.41	45±1.43	52±1.78
Holding time	40	27±0.83	35±0.75	40±1.35
(min)	80	23±0.83	32±0.51	33±0.79

* Numbers represent the values of the mean ± SEM

Water vs. culture medium

Survival also depended on whether the cryoprotectants were dissolved in water or culture medium (Table 7). Recovery was higher when the cryoprotectants were prepared in water, and this was true for both the cell lines.

Uptake time

Viability was not improved by leaving the cells in the cryoprotectant solution for one hour before freezing (Table 8).

Protoplast isolation

When protoplasts were isolated from both control and cryopreserved cells, the yields determined by hemacytometric counting varied, the cryopreserved cells giving a much higher yield than the control. Similarly, when the protoplasts were plated in culture medium, although a density of $1\text{-}3x10^5$/ml was used with both, the plating efficiency of cryopreserved cells was again found to be higher than the control (Table 9).

When *Panicum maximum* cells were cryopreserved using all the optimum parameters, the survival obtained was 99% according to TTC test and 103% according to regrowth potential (Table 10; Fig. 42). Survival based on regrowth potential was obtained from fresh weights on day 4 and day 12. When treated with cryoprotectant there was a slight reduction in viability when the cells were returned to culture. Both rapid and slow thawing were successful for cryopreservation of *Panicum maximum* cells, rapid thawing being slightly better. No reduction in viability was observed in any of the stages. No major reduction in viability was observed in cells of *Panicum maximum* stored in liquid nitrogen for up to three years.

Table 7: Survival of *P. maximum* and *P. americanum* cells when cryoprotected with compounds made in water vs. culture medium (averages of two experiments, five replicates per experiment).

	% survival*	
	P. maximum	*P. americanum*
water	85 ± 0.57	51 ± 1.86
Culture medium	74 ± 1.52	43 ± 0.73

* Numbers represent the values of the mean ± SEM

Table 8: Effect of cryoprotectant uptake time on survival of *P. maximum* and *P. americanum* cells. (averages of two experiments, five replicates per experiment).

Time(hr)	% survival*	
	P. maximum	*P. americanum*
0	85 ± 0.57	53 ± 2.00
1	82 ± 0.97	51 ± 1.64

* Numbers represent the values of the mean ± SEM

Table 9: Protoplast yields and plating efficiencies (PE) of cryopreserved and control cells of *P. maximum*.

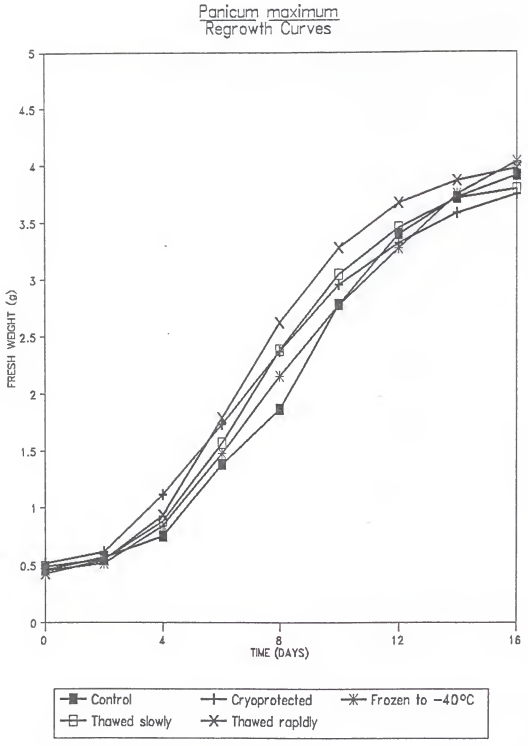
	Cryopreserved cells		control cells	
	Yield	PE	Yield	PE
Expt. 1	$25x10^6$	51%	$16x10^6$	33%
Expt. 2	$16.2x10^6$	58%	$7.7x10^6$	34%

Table 10: Survival of cells at different stages of the cryopreservation procedure (averages of two experiments, five replicates per experiment).

Stage	% survival*			
	P. maximum		*P. americanum*	
	TTC	RP	TTC	RP
After cryoprotection	98 ± 0.71	83 ± 4.22	86 ± 0.85	105 ± 2.1
-40°C	101 ± 1.07	91 ± 3.26	55 ± 0.84	82 ± 3.00
Rapid thawing	99 ± 1.66	103 ± 4.17	54 ± 1.66	92 ± 2.64
Slow thawing	97 ± 0.75	97 ± 3.73	30 ± 0.82	00 ± 0.00

* Numbers represent the values of the mean ± SEM

Fig. 42. Regrowth curves of *Panicum maximum* cells at different stages of the cryopreservation procedure.


Panicum maximum
Regrowth Curves
FRESH WEIGHT (g)
TIME (DAYS)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
4
8
12
16
Control
Cryoprotected
Frozen to -40°C
Thawed slowly
Thawed rapidly

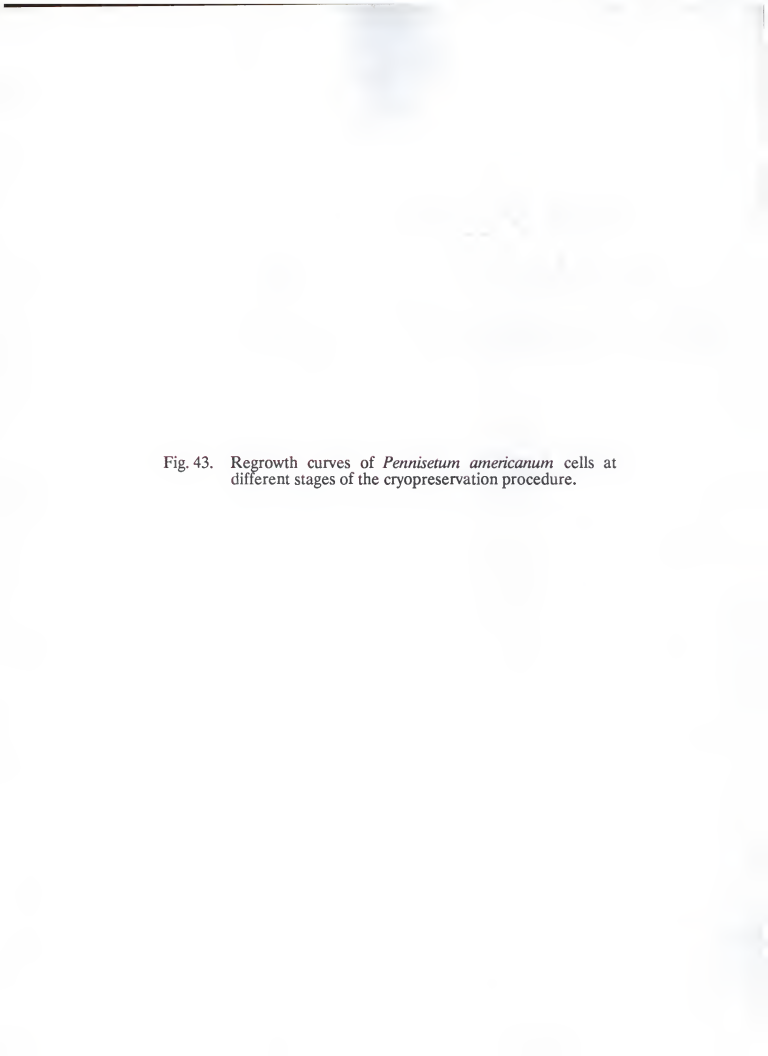
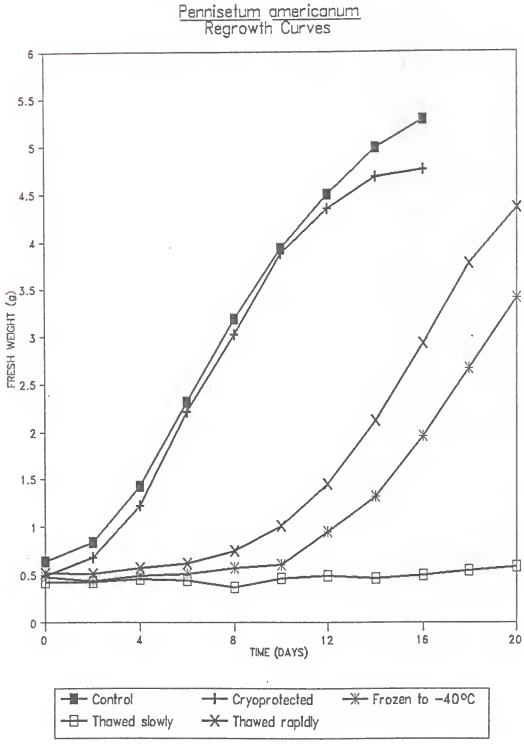


Fig. 43. Regrowth curves of *Pennisetum americanum* cells at different stages of the cryopreservation procedure.


Pennisetum americanum
Regrowth Curves
FRESH WEIGHT (g)
TIME (DAYS)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
0
4
8
12
16
20
Control
Cryoprotected
Frozen to -40°C
Thawed slowly
Thawed rapidly

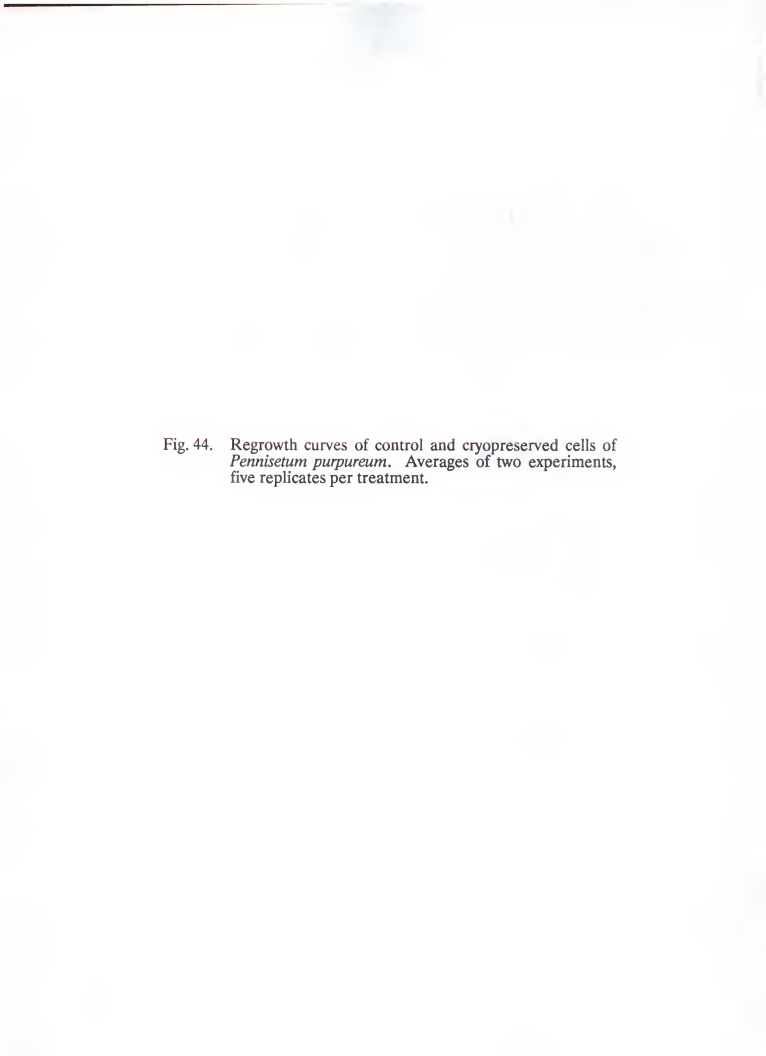


Fig. 44. Regrowth curves of control and cryopreserved cells of *Pennisetum purpureum*. Averages of two experiments, five replicates per treatment.

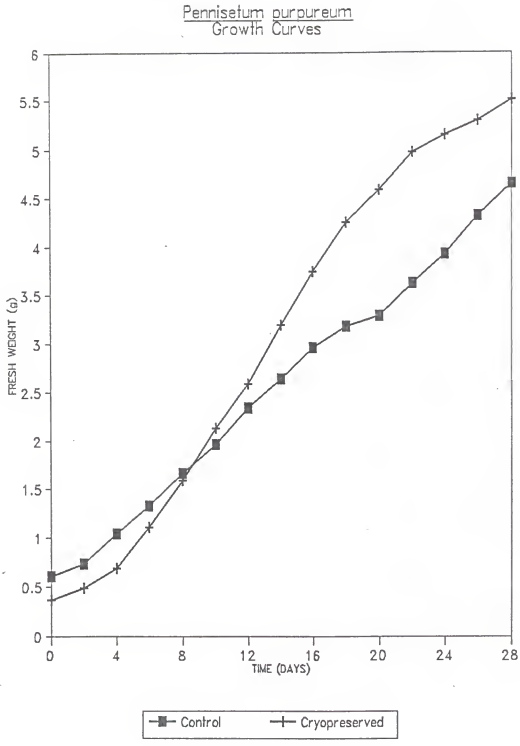

Pennisetum purpureum
Growth Curves
FRESH WEIGHT (g)
TIME (DAYS)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
0
4
8
12
16
20
24
28
Control
Cryopreserved

Fig. 45. Regrowth curves of control and cryopreserved cells of *Saccharum* hybrid (SH2). Averages of two experiments, five replicates per experiment.

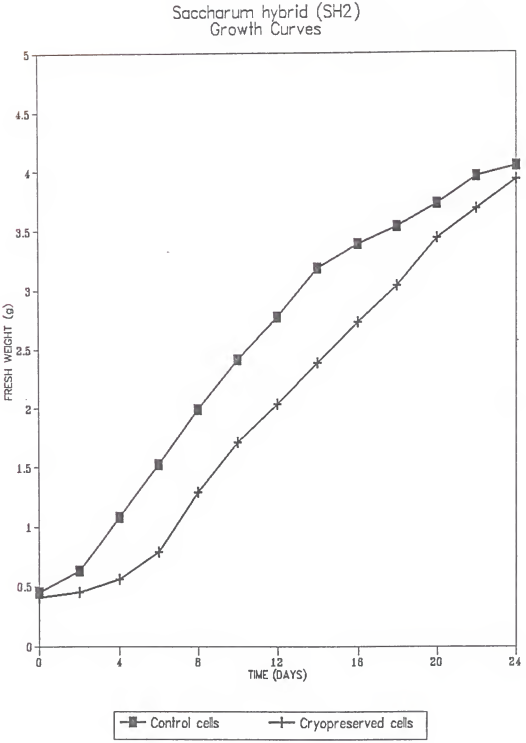
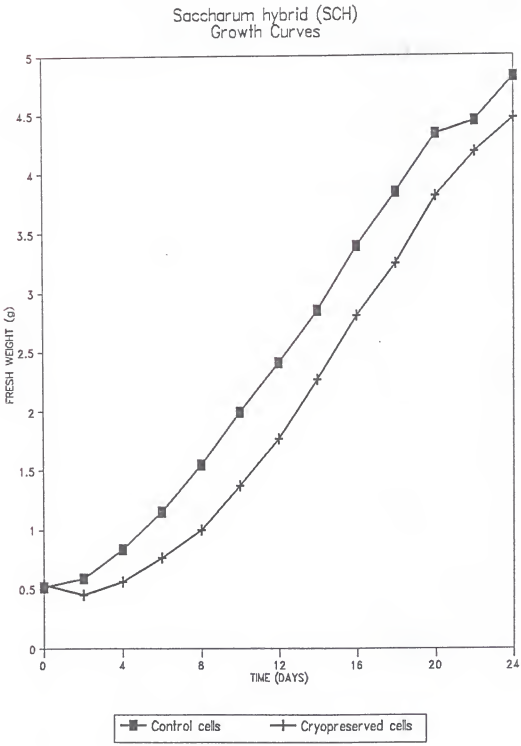

Saccharum hybrid (SH2)
Growth Curves
FRESH WEIGHT (g)
TIME (DAYS)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
4
8
12
16
20
24
Control cells
Cryopreserved cells

Fig. 46. Regrowth curves of control and cryopreserved cells of *Saccharum* hybrid (SCH). Averages of two experiments, five replicates per experiment.


Saccharum hybrid (SCH)
Growth Curves
FRESH WEIGHT (g)
TIME (DAYS)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
4
8
12
16
20
24
Control cells
Cryopreserved cells

With *Pennisetum americanum* cells slow thawing was lethal. Although a 20% viability was obtained according to TTC test, the cells failed to resume growth when returned to culture (Table 10; Fig. 43). Low survival was also observed with cells cooled to -40°C and cells frozen to liquid nitrogen temperatures and thawed rapidly based on TTC reduction assay. When cryopreserved cells were returned to culture the cells resumed growth after a lag period of about ten days. Hence growth rates were measured between day 4 and day 10 for control cells and cells treated with the cryoprotectants and plated without freezing. For cells with a lag phase the growth rate was measured between day 14 and day 20.

Survival rates of 162%, 91% and 96% were obtained with cell suspensions of *Pennisetum purpurium* and *Saccharum* hybrids (SCH and SH2), respectively, when frozen using the optimized cryopreservation procedure (Figs. 44-46).

Discussion

Survival of cell suspensions depends on a number of factors during the cryopreservation process. Age, nature and physiological state of the cells determine the ability of the cells to withstand the freezing process. Cells in the late lag or early exponential phase are more resistant to freeze damage than those in the late exponential or stationary phase (Sugawara and Sakai 1974; Withers and Street 1977a,b). It has also been reported that small highly cytoplasmic cells survive better than large highly vacuolated cells (Nag and Street 1975a). The decrease in resistance to freezing in the stationary phase is due to the occurrence of increase in cell size associated with higher vacuolation and water content, which cause the cells to be more susceptible to freezing injury due to plasmolysis (Withers and Street 1977a).

Freeze tolerance can be increased by pregrowing the cells in a high osmotic medium supplemented with such additives as mannitol, sorbitol, various sugars or proline prior to freezing (Latta 1971; Bannier and Steponkus 1972; Withers and King 1979a,b; Pritchard *et al.* 1986). During freezing, plasmolysis is the major cause of injury in highly vacuolated cells (Withers and Street 1977a), and various osmotic compounds can minimize this damage by exerting forces that inhibit cell expansion and thereby reducing the volume of the cell and its vacuole. The water content of the cell is reduced by these changes, hence requirement for protective dehydration is reduced during freezing (Withers 1985b,c).

When cells were pregrown in high osmoticum, reduction in cell volume, cytoplasm to vacuole ratio, cellular water content and accumulation of cytoplasmic solutes change the osmotic behavior of the cells making them more resistant to freeze damage (Withers 1985b).

Mannitol is the most commonly used pregrowth additive, and concentrations up to 6% have been employed (Withers and King 1980). Pregrowing the cells in sorbitol has also been effective (Chen *et al.* 1984a,b; Kartha *et al.* 1988), and improved survival in instances where mannitol was not successful (Maddox *et al.* 1983).

Pregrowing cells of *Panicum maximum* and *Pennisetum americanum* for three days in a medium supplemented with mannitol or sorbitol improved their viabilities considerably. It has been suggested that cells should be pregrown in supplemented medium for at least 3-4 days to get positive response, but prolonged exposure may cause darkening of the cell and reduction in viability (Withers 1983).

Cell damage occurs during the process of freezing due to solution effects resulting from excessive dehydration, or by ice damage from formation of intracellular ice crystals (Mazur *et al.* 1972). Cryoprotectants which are applied prior to freezing, reduce freeze injury in a number of ways. Penetrating compounds

act by causing depression of freezing point of the cytoplasm and also provide an increased volume to reduce damage caused by increased concentration of toxic solutes and electrolytes in the cytoplasm (Finkle *et al.* 1985b). Non-penetrating compounds act by osmotic action reducing the water content of the cell, thereby exerting protective dehydration and preventing ice damage (McGann 1978).

Using a mixture of cryoprotectants at low concentrations is superior to using a single compound at high concentrations, because of the additive cryoprotective effect obtained along with a reduction in individual toxicity (Finkle and Ulrich 1978, 1979; Bajaj 1983; Bhandal *et al.* 1985; Finkle *et al.* 1985b). Two combinations which are often used and found to be successful in a number of studies, 0.5 M sorbitol and 5% DMSO (Chen *et al.* 1984a,b; Kartha *et al.* 1988) and 0.5 M DMSO, 0.5 M glycerol and 1 M sucrose were used for cryopreservation of both cell lines. Cryoprotectants should be added to the cell suspension in small increments, as sudden addition may cause plasmolysis (Towill and Mazur 1976). Addition should be carried out at low temperatures to reduce the toxic effects of the cryoprotectants. DMSO is toxic at higher temperatures because of its temperature dependent interactions with proteins (Arakawa *et al.* 1990).

High concentrations of cryoprotectants or long term exposure may damage the cells (Dougall and Wetherell 1974; Nag and Street 1975a; Bajaj and Reinert 1977). Leaving cells of *Panicum maximum* and *Pennisetum americanum* for an hour at 0-4°C after cryoprotectant addition to facilitate uptake did not improve survival. Therefore, it is best to freeze the cells immediately after the addition of cryoprotectants without a period of uptake.

Cell cultures can be frozen either rapidly or slowly to liquid nitrogen temperatures. During slow cooling, increase in the concentration of the solution occurs due to formation of extracellular ice and the subsequent removal of water from the cell. If the cooling rate is too slow, all the freezable water leaves the cell

causing dehydration injury. If the rates are too rapid protective dehydration is prevented from taking place, causing damage due to intracellular ice formation. The optimal cooling rate should be such that it is slow enough to prevent intracellular ice formation but not too slow to cause any dehydration damage (Meryman and Williams 1985).

Cell suspensions are generally frozen slowly, and rates of 0.5°C/min to 4°C/min have been reported to be optimal (Withers and Street 1977a; Withers 1984a). When cells of *Panicum maximum* and *Pennisetum americanum* were frozen slowly, highest recovery was obtained at a cooling rate of 0.5°C/min.

Slow freezing is generally terminated at a temperature in the region of -40°C, and cells are plunged into liquid nitrogen after a holding period of up to one hour (Withers 1985b). With *Panicum maximum* and *Pennisetum americanum* a transfer temperature of -20°C gave very poor survival rates. The damage may probably be due to inadequate dehydration at this temperature which will cause intracellular ice crystallization when transferred to liquid nitrogen. A transfer temperature of -40°C gave the best results, while cooling to lower temperatures caused a reduction in viability. In addition, a holding period at the transfer temperature also had adverse effects on viability. Cooling to too low a transfer temperature or prolonged exposure to the transfer temperature may be injurious to cells by the occurrence of excessive cellular dehydration (Withers 1985c; Kartha *et al.* 1988). Therefore, it is best to cool the suspensions to a transfer temperature of -40°C and plunge immediately into liquid nitrogen without a holding period.

Survival of *Panicum maximum* and *Pennisetum americanum* cells increased with increase in PCV. Bajaj (1976a) reported that ampoules containing thick suspension gave better survival compared to ones with low cell density. Generally a minimum cell density is required for cells to resume growth (Stuart and Street 1969; Sala *et al.* 1979), and resumption of growth is adversely affected if the cell numbers

fall below this value. Therefore, it is important to concentrate the cells prior to freezing to compensate for the loss of cells that occurs during freezing and thawing. When very low survival rates were obtained from TTC test, the cells failed to resume growth on culture medium (Tables 1, 2, 10). This could be due to the reduction of the cell density of the viable cells below the minimum density required for resumption of growth.

Viabilities were determined by both TTC reduction assay and regrowth potential. TTC test is used to get a rapid estimation of viability and has been proven to be reliable in a number of studies (Sugawara and Sakai 1974; Towill and Mazur 1976; Finkle and Ulrich 1979). It is based on the ability of the cell to reduce tetrazolium salts to formazan by mitochondrial activity (Steponkus and Lanphear 1967). Formazan is a water insoluble pigment which can be extracted in alcohol. The amount of formazan produced can be determined spectrophotometrically by measuring the absorbance at 530 nm, and is proportional to the number of viable cells present. In this study a positive correlation was observed between viabilities obtained from TTC test and regrowth potential. However, under-estimation was evident, especially with *Pennisetum americanum* cells. TTC test was used to get a rapid estimation of viability. It was used to compare viabilities between different treatments of the same experiment, and never to compare different experiments. The final results were verified by using regrowth potential which depends on the ability of the cells to resume growth in culture.

Viabilities obtained from the TTC test were similar to those obtained from regrowth potential for *Panicum maximum* cells. When viabilities were determined at different stages of the cryopreservation procedure not much loss of viability occurred at any of the stages. This was again confirmed from the regrowth potential assay. Cells from all stages resumed growth without a lag period and grew at rates comparable to that of the control.

In contrast, with *Pennisetum americanum* cells, differences were observed in the viability of the cells during the cryopreservation procedure. Viability was reduced to 86% of the control cells after treatment with the cryoprotectants prior to freezing. When these cells were plated on semi-solid medium they resumed growth without a lag period and grew as well as the control cells. DMSO, which is the most widely used cryoprotectant, is known to reduce respiration and inhibit RNA and protein synthesis at high concentrations (Bajaj *et al.* 1970; Morris 1976; Kartha *et al.* 1982). Since the TTC test estimates viability from the mitochondrial activity the reduced viabilities obtained from the TTC test may be due to the reduced mitochondrial activity caused by DMSO. Cells thawed after freezing to -40°C gave a viability of 55% according to the TTC test. But on culture medium they resumed growth after a lag period of about 10 days. Therefore the reduced viabilities that were observed with the *Pennisetum americanum* cells with the TTC test could have been the result of the lag phase that immediately followed thawing. Cells which are partially injured or in a cold shock state may not give a positive reaction with the stain immediately after thawing but later revive in culture (Bajaj 1976a, 1979a). Cella *et al.* (1982) reported that a number of physiological and biochemical alterations occur during the process of cryopreservation, and growth resumes after a lag phase during which time these damages are repaired.

Panicum maximum cells were not affected by the rapid and slow thawing method used, whereas *Pennisetum americanum* cells resumed growth only when they were thawed rapidly. Rapid thawing is mostly employed for cell suspensions (Withers 1985b). Recrystallization of ice is a major cause of injury during thawing due to the formation of progressively larger ice masses. The zone of recrystallization is passed so rapidly during rapid thawing that there is no time for recrystallization to take place, hence cell injury is prevented. If the cells are cooled to transfer temperatures beyond -40°C, viabilities are not affected by the thawing

method employed due to the optimum dehydration that has taken place (Withers 1985b; Bhojwani and Rhazdan 1983). No differences in viability between rapid and slow thawing rates were observed with carrot and sycamore cell suspensions after cryopreservation (Dougall and Wetherell 1974; Sugawara and Sakai 1974). Therefore, it is best to employ rapid thawing in cryopreservation of cell suspensions. Even with *Panicum maximum*, where high survival was obtained with slow thawing, the rates were not much higher than when rapid thawing was used.

The cell suspension of *Panicum maximum* gave improved protoplast yield and plating efficiency after cryopreservation. Embryogenic suspension cultures comprised primarily of small, cytoplasmic cells are ideal for cryopreservation because of the absence of any large vacuoles. The few large vacuolated cells present do not survive cryopreservation. This can be a useful selection process for embryogenic cells. Shillito *et al.* (1989) reported that in maize, callus recovered from cryopreserved suspension could by used to establish new suspensions that can be used more efficiently for protoplast isolation and gave improved protoplast plating efficiencies. This procedure can, therefore, enrich a cell line with embryogenic cells, albeit for a short period of time since the suspension normally reverts to a heterogeneous population.

The optimized procedure for cryopreservation of *Panicum maximum* and *Pennisetum americanum* was used successfully for the cryopreservation of a number of Gramineous species. Preliminary experiments were not necessary. No loss of viability was observed in cells that were in storage for up to three years. During the process small highly cytoplasmic cells are selected over large highly vacuolated cells, thereby enriching the suspension with embryogenic cells which ware more desirable for protoplast isolation and transformation.

CHAPTER 5

CRYOPRESERVATION OF AN EMBRYOGENIC CALLUS CULTURE AND A CELL SUSPENSION CULTURE OF A COMMERCIAL *SACCHARUM* HYBRID

Introduction

In most species of the Gramineae, efficient and reliable plant regeneration can be obtained from embryogenic tissue cultures. The establishment and maintenance of embryogenic cell suspension cultures of cereals and grasses have, however, proven to be extremely difficult. Such cultures have been obtained in only a few species such as maize, rice, pearl millet, and sugarcane (Vasil 1988), and are of considerable importance since they are currently the only source of totipotent cells and protoplasts for genetic transformation studies of this important group of crop plants (Vasil 1988; Potrykus 1990).

Callus cultures, essential for the establishment of cell suspension cultures are initiated from young leaves, inflorescence, and immature embryos. In tissue culture of cereals, callus cultures offer the most efficient means of regeneration (Vasil and Vasil 1986). Routine maintenance of callus is a technically demanding process in which suitable type of callus is selected and transferred to fresh medium at appropriate time intervals. Regeneration potential of callus cultures declines with time, and eventually the tissue looses the capacity to regenerate altogether. It will be very useful, therefore, if regenerating callus as well as cell suspension cultures could be cryopreserved.

Materials and Methods

Sugarcane (*Saccharum* sp. hybrid) plants of cv CP72-1210 (CP65-357 x CP56-63) were obtained from Dr. J.D. Miller (USDA Sugarcane Experimental Station, Canal Point, Florida), and were grown in a greenhouse. Shoots were cut just below the youngest visible node. The outermost leaves were removed and the remaining shoot was trimmed and cleaned with 95% ethanol until slightly bleached. All but the innermost 4-6 leaves were removed aseptically in a laminar flowhood. Cultures were initiated by cutting 2 mm thick transverse segments of the young leaf bases and placing them on semi-solid (0.2% Gelrite) MS basal medium (Murashige and Skoog 1962) supplemented with 3% sucrose, 3 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 100 mg/l inositol and 5% coconut water (MS3C medium), and the pH was adjusted to 5.8 before autoclaving. No portion of the stem was included in the explant. Cultures were then incubated in the dark at 28°C. The explants were transferred to fresh medium every two days until discoloration of the medium stopped.

As reported in previous work by Ho and Vasil (1983), two types of calli were formed from the leaf segments in seven to ten days: a white compact nodular embryogenic callus, and a soft friable non-embryogenic callus. After four weeks the white compact embryogenic calli, which could be easily distinguished from the non-embryogenic calli, were selected and transferred to fresh MS3C medium. Routine maintenance was carried out by selecting and transferring the embryogenic calli to fresh MS3C medium at monthly intervals, and a nine to twelve month old callus culture was used for cryopreservation experiments.

Embryogenic calli formed from the leaves were also used for the establishment of an embryogenic cell suspension. Pieces of embryogenic calli (1-1.5 g) were selected and placed in 10 ml of liquid MS3C medium in 125 ml

Erlenmeyer flasks. The supernatant was replaced weekly with an equal volume of fresh medium. After 3-4 months they were transferred to 250 ml Erlenmeyer flasks containing 35 ml of fresh medium. A homogeneous cell suspension was formed in about twelve months. The cell line was routinely maintained by transferring 12 ml of the cell suspension to 35 ml of MS2C medium, and was used for cryopreservation.

Cryopreservation of Cell Suspension

Pregrowth conditions

The following five pregrowth conditions were tried.

1. MS2C
2. MS2C + 6% (w/v) mannitol
3. MS2C + 6% (w/v) sorbitol
4. MS2C + 5% (v/v) DMSO
5. MS2C + 0.20 M sorbitol for 24 hours followed by 0.40 M sorbitol for another 24 hours (Kartha *et al.* 1988).

Twelve ml of the seven day old cell suspension culture was transferred directly to 35 ml of the pregrowth media, and the cells were used for cryopreservation on the fourth day after subculture (Except for treatment 5).

Cryoprotectant treatment

A 10 ml aliquot of the cell suspension was centrifuged at 100 x g for three minutes. The packed cell volume was adjusted to 80% of the original volume, and then the suspension was chilled on ice. An equal volume of cryoprotectants (Table 11), prepared in double strength, filter sterilized, and chilled on ice was added to the cell suspension culture over a period of one hour with agitation. The suspension

was agitated on a rotary shaker for one more hour while being maintained on ice to facilitate the uptake of the cryoprotectants.

Freezing

One ml of the cell suspension was transferred to each of the 1.2 ml polypropylene, screw-cap, cryogenic ampoules. These were placed in a Cryomed Model 1010 Micro Computer Programmable Freezer Unit, and frozen at a rate of 0.5°C/min to -40°C. The cooling rate was monitored by placing the temperature probe in one of the ampoules. After 45 minutes at -40°C they were stored in liquid nitrogen.

Cells were also frozen without any pregrowth or cryoprotective treatment by plunging them directly into liquid nitrogen or transferring them to liquid nitrogen after bringing the temperature down to -40°C. Cells treated with cryoprotectants were also frozen by immersing them directly into liquid nitrogen in polypropylene ampoules or in sterile Petri dishes.

Thawing and Regrowth

After one month of storage in liquid nitrogen, the ampoules were thawed rapidly by swirling in a 40°C water bath until the ice had just disappeared (approximately 45-60 seconds), and then transferred immediately to an ice bath to prevent excessive rise of temperature. The thawed cell suspension was then plated on the following three media for regeneration.

1. MS basal + 3% sucrose (MS0)
2. MS0 + 1 mg/l BAP (benzyl aminopurine) (MSB)
3. MS0 + 0.13 mg/l 2,4-D + 0.25 mg/l BAP + 0.25 mg/l kinetin + 0.25 mg/l zeatin (MSSR).

MSB and MSSR have both been used previously in our laboratory for efficient regeneration of plants from leaf-derived embryogenic callus cultures of sugarcane (F. Morrish and A. El Bakry, personal communications).

The cryoprotectants were removed by two methods, *i.e.* 1) washing and 2) filter paper discs. In the washing method the cryoprotectants were gradually diluted with MS2C medium. The contents of an ampoule were transferred to a sterile tube and the volume was brought to 10 ml over a period of 30 minutes with liquid MS2C. The cells were centrifuged and then transferred to semi-solid regeneration media. In the other method filter paper discs were used to remove the cryoprotectants without washing the cells (Kartha *et al.* 1988). The cells were allowed to settle and the supernatant was gently poured off before plating the cells on filter paper discs placed on semi-solid regeneration medium. After five hours the filter paper discs along with the cells were transferred to fresh medium. Finally, the cells were scraped off after 24 hours and plated on fresh medium. The cultures were kept at 28°C in the dark for two weeks before transfer to light.

Viability assay

Cell survival was determined by using TTC reduction assay procedures (Steponkus and Lanphear 1967). The absorbance of the solution was recorded at 530 nm using a Beckman DU-40 Spectrophotometer and expressed as percent survival over the control (Kartha *et al.* 1982):

$$\frac{\text{TTC value of frozen cells}}{\text{TTC value of unfrozen control cells}} \times 100$$

For a more reliable test of viability, plants produced from 1 ml of plated cell suspension were counted.

Shoots formed in regeneration media were transferred to rooting medium (MS basal + 3% sucrose + 0.2 mg/l NAA (alpha- naphthaleneacetic acid) in Magenta boxes. Activated charcoal (10 g/l) was added to some of the regeneration media. After an extensive root system was formed, the plants were transferred to soil in Conetainers (Ray Leach Conetainer Nursery) for a month and then transferred to the greenhouse .

Cryopreservation of Callus Culture

Pregrowth treatments

Pregrowth treatments were not used for cryopreservation of callus cultures in most of the experiments. In one experiment, the callus cultures were grown in semi-solid MS3C medium supplemented with 5% DMSO for 1-3 days before cryopreservation.

Cryoprotectant treatment

Nine to twelve month old callus cultures were used for cryopreservation. Embryogenic callus pieces about 1-3 mm in size were selected from 10-15 day old cultures, and incubated in the filter sterilized, pre-chilled cryoprotectant solution for one hour at 0°C with stirring, and transferred to polypropylene ampoules for freezing. Calli were also treated with cryoprotectants by transferring to prechilled liquid MS3C medium, and then adding prechilled cryoprotectants prepared in double strength, over a one hour period. The tissue was then incubated for one more hour at 0°C with agitation, before transfer to the ampoules.

Different cryoprotectants were used either singly or in combination.

1. 5% DMSO
2. 0.5 M sorbitol + 5% DMSO

3. 0.5 M DMSO + 0.5 M glycerol + 1 M sucrose
4. 10% polyethylene glycol (PEG) + 8% glucose + 10% DMSO
5. 5% Glucose + 10% DMSO

Freezing

After treatment with cryoprotectants, the callus tissue was frozen by different methods.

1. With slow freezing, a cooling rate of 0.5°C/min was used for all experiments. The procedure for freezing of callus cultures was the same as for cell suspension cultures. One ml of the cryoprotectant solution along with the callus was dispensed into each of the 1.2 ml cryogenic ampoules, and frozen at a rate of 0.5°C/min to -40°C, held at this temperature for another 45 minutes before plunging into liquid nitrogen.
2. Calli were removed from the cryoprotectant solution, blotted dry and transferred to cryogenic vials and frozen as in treatment 1.
3. Calli were removed from the cryoprotectant solution as in treatment 2, but frozen by direct immersion in liquid nitrogen.

Some of the calli were removed from the cryoprotectant solution before freezing to determine any toxic effects of the cryoprotectants on the callus tissue. Equal volumes of callus tissue from each treatment, and control tissue without cryoprotectant addition or freezing, were used to obtain a photographic record. Callus removed from each treatment before freezing were blotted dry and plated on semi-solid MS3C medium, along the periphery of a Petri dish, and the control tissue was plated at the center. A photographic record was kept of these tissues by taking pictures at monthly intervals (Figs. 51, 52).

Thawing and Regrowth

Rapid thawing was employed as with the cell suspension. Calli which were frozen by treatment 1 were plated by the following methods.

A. The calli were plated without washing by plating directly on semi-solid medium with the cryoprotectants, and after gradual removal of cryoprotectants by the filter paper technique used for the suspension culture.

B. The calli along with the cryoprotectant solution were transferred to a 15 ml centrifuge tube, and the volume was brought up to 10 ml by gradual addition of liquid MS3C medium at 0°C.

C. Same as B, but the dilution was done at room temperature.

Calli from treatment 2 and 3 were blotted dry and plated on semi-solid MS3C medium. Survival of the callus tissue was determined by regrowth potential.

The cryopreserved tissues were also plated on the semi-solid MS3C medium with the control cells to obtain a photographic record. The callus at the center of the dish was plated without freezing or cryoprotective treatment, and the tissue from each treatment was plated along the periphery of the Petri dish. For each treatment the tissue was plated with and without washing. The tissues were photographed immediately after plating (Fig. 53) , and one month later (Fig. 54), after which the calli were transferred to fresh medium. With cryopreserved tissue due to the lag period which occurred after cryopreservation all of the tissue was transferred to fresh medium, whereas with control tissue the same amount that was used originally was again plated at the center. The Petri dish was photographed again after one month (Fig. 55). Once the embryogenic calli started to form, they were maintained by selection and transfer to fresh MS3C medium and to regeneration medium. The calli on MS3C medium were maintained at 27°C in total darkness, whereas the ones

on regeneration medium were incubated in light immediately upon transfer or after two weeks in total darkness.

Callus cultures cryopreserved using 5% DMSO as cryoprotectant were plated by the three methods mentioned previously. Four hundred mg of tissue was used in each experiment and the fresh weight increase was compared to the control by determining the increase in fresh weight after one and two months.

Callus cultures cryopreserved by the different methods discussed previously, differed in their response. But even with the treatments that gave optimal result the cultures started to grow after an extended lag period, and failed to regenerate plants. To overcome this, a pregrowth period was introduced into the procedure. Embryogenic callus tissue was selected from a 10-15 day old culture, and suspended into 35 ml of MS2C medium supplemented with 6% sorbitol in a 250 ml Erlenmeyer flask. Sorbitol was selected as the pregrowth additive since it gave optimal result with the suspension culture. The callus cultures were left for two days in this high osmotic medium on a rotary shaker in total darkness.

The callus tissues were then concentrated by removing excess medium, and chilled on ice. Equal volume of cryoprotectant prepared in double strength (10% DMSO), was added next over a one hour period with agitation, and the tissues were left for one more hour on a rotary shaker at reduced temperature. The callus tissue along with the cryoprotectant solution was transferred to ampoules and frozen slowly as described previously before storing in liquid nitrogen.

Thawing was done rapidly and the callus tissue was plated on semi-solid MS3C medium for regrowth as well as on MSB and MSSR media for regeneration. The regenerated plants were ready to be transferred to rooting medium after two months.

Results

Cell Suspension Culture

Normally there is a good correlation between the values for cell viability calculated from the TTC test and the plating efficiency. This was not the case in cryopreservation of sugarcane cell suspension (Table 11). According to the TTC test, the highest survival was obtained when the cells were pregrown in MS2C + 6% sorbitol and cryopreserved with 0.5 M glycerol, 0.5 M sorbitol and 1 M sucrose. No plants were obtained, however, in any of the experiments where this specific combination of chemicals was used as cryoprotectant. When placed on semi-solid regeneration medium, the cells which had looked healthy for the first 2-3 days, turned brown and died. Although these cryoprotectants have been found to be very useful for protecting cells from freeze damage in a variety of species, they were not suitable for sugarcane cells. To determine whether the death of cells was due to toxic effects caused by the cryoprotectants, or the inability to prevent freeze damage during freezing, the cells were pregrown in MS2C + 6% sorbitol and MS2C + 6% mannitol and treated with 0.5 M glycerol, 0.5 M DMSO, and 1 M sucrose as described before. After uptake of cryoprotectants for one hour on a rotary shaker, the cells were plated on MSB , and MSSR media without freezing. The cells showed similar growth to the control (cells plated without any pretreatment or cryoprotection). Therefore, the death of the cells must have been the result of freeze damage when this particular combination of cryoprotectants were used.

Washing thawed cryopreserved cells was deleterious in all treatments. Browning was suppressed in washed cells but no further growth was observed. When the cells were plated directly on semi-solid medium without removing the cryoprotectants, extensive browning of the tissue occurred. Healthy tissue was

Table 11: Effect of cryoprotectants on the survival of sugarcane cell suspension cultures (averages of two experiments. There were five replicate plates for each treatment, with 1 ml cells/plate).

Pregrowth	Cryoprotectants	Percent survival*	Percent Regeneration*	
		TTC	MSB	MSSR
MS2C	0.5 M sorbitol + 5% DMSO	15±0.69	4±5.30	18±5.59
	0.5 M glycerol + 0.5 M DMSO + 1.0 M sucrose	27±1.30	00±0.00	00±0.00
MS2C + 6% mannitol	0.5 M sorbitol + 5% M DMSO	29±0.78	5±3.04	22±6.43
	0.5 M glycerol + 0.5 M DMSO + 1.0 M sucrose	96±1.02	00±0.00	00±0.00
MS2C + 6% sorbitol	0.5 M sorbitol + 5% DMSO	90±0.39	74±5.09	92±2.94
	0.5 M glycerol + 0.5 M DMSO + 1.0 M sucrose	132±2.75	00±0.00	00±0.00
MS2C + 5% DMSO	0.5 M sorbitol + 5% DMSO	19±0.73	00±0.00	00±0.00
	0.5 M glycerol + 0.5 M DMSO + 1.0 M sucrose	33±1.37	00±0.00	00±0.00
MS2C + 0.2 M sorbitol MS2C + 0.4 M sorbitol	0.4 M sorbitol + 5% DMSO	80±1.81	45±4.12	70±3.53

* Numbers represent the values of the mean ± SEM

formed when the cryoprotectants were removed gradually using filter paper discs. Some discoloration was observed, but this was comparable to the unfrozen control. The recovered tissue was transferred to fresh medium for further growth.

All three media (MS0, MSB and MSSR) were successfully used for the regeneration of plants from the original cell suspension. MS0 did not, however, prove to be useful for the regeneration of cryopreserved cells, as callus growth was followed by profuse root formation after four weeks. In MSB medium shoots were formed in four weeks, but were allowed to remain in the same medium for an additional four weeks to facilitate better shoot development. Shoots were subsequently transferred to rooting medium for a month during which time an extensive root system developed. In MSB medium, plants recovered from the plated cryopreserved cell suspension could be transferred to soil in three months. In MSSR medium, shoots also developed in four weeks (Fig. 47). They were, however, maintained in the same medium for a total of three months, because growth was considerably slower, although the number of shoots produced was much higher than in MSB. Plants were then transferred to rooting medium for a month (Fig. 48, 49), and then to soil (Fig. 50). A total of four months was needed for the production of plantlets that were ready to be potted.

In all instances the addition of activated charcoal was harmful to shoot development (Table 12). In the absence of activated charcoal, each piece of callus formed many plants. When charcoal was added only one or two plants were produced. Furthermore, the plants were etiolated (Fig. 48).

When thawed and plated, the cells that were frozen without any cryoprotective treatment, showed neither browning nor growth. Cells treated with cryoprotectants and then plunged into liquid nitrogen in sealed ampoules did not grow at all, but the cells treated in the same manner and frozen in Petri dishes

Fig. 47. Regeneration of a cryopreserved cell suspension of sugarcane on MSSR medium.

Fig. 48. Effect of activated charcoal on regeneration of a cryopreserved cell suspension of sugarcane. With charcoal (L), without charcoal (right).

Fig. 49. Plantlets formed from cryopreserved cells in rooting medium.

Fig. 50. Mature plants from cryopreserved (left), and control (right) cell suspensions.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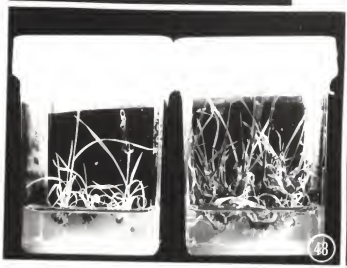
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Table 12: Effect of activated charcoal on regeneration of sugarcane suspension cultures (averages of two experiments. There were five replicate plates for each treatment, with 1 ml cells/plate).

Treatment	Cryopro-tectants	Percent Regeneration*			
		MSB		MSSR	
		charcoal	no charcoal	charcoal	no charcoal
MS2C + 6% sorbitol	0.5 M + sorbitol + 5% DMSO	18±6.60	74±5.09	11±1.13	92±2.94

* Numbers represent the values of the mean ± SEM

Table 13: Increase in fresh weight of cryopreserved and control callus cultures of sugarcane (Averages of two experiments).

	Initial weight (g)	After one month (g)	After two months (g)
Control	0.40	2.079	2.130
	0.40	2.434	2.609
Without washing	0.40	0.702	2.119
	0.40	0.732	2.424
Washed at 0°C	0.40	0.484	1.309
	0.40	0.413	1.314
Washed at RT	0.40	0.352	1.040
	0.40	0.225	0.921

showed good growth but contamination was a consistent problem that could not be eliminated.

The highest number of plants were produced when cells were pregrown in MS2C medium supplemented with 6% sorbitol and cryopreserved with 0.5 M sorbitol and 5% DMSO . Fifty plants regenerated from this combination of treatments were grown to maturity in the greenhouse. These plants were morphologically identical to the controls.

Callus Cultures

The growth of embryogenic callus cultures treated with different cryoprotectants and plated without freezing was found to be similar to each other and to that of the control (Figs. 51, 52). There was no lag period and growth resumed immediately after plating. When cryopreserved tissues were plated in the same way (Fig. 53), however, marked differences in growth were observed. Very little growth was observed during the first month. In fact the only cryopreserved tissues that resumed growth were the ones cryopreserved with 5% DMSO, and 0.5 M sorbitol + 5% DMSO and plated without washing (Fig. 54). After one more month in culture, differences in response of the callus to different cryoprotectants and the deleterious effects of washing could be clearly seen (Fig. 55). Highest survival was obtained when 5% DMSO was used. The 0.5 M sorbitol + 5% DMSO treatment also gave similar results. A lag period of 3-4 weeks was observed with both treatments, during which time very little growth took place, but no browning was observed. The callus tissue that formed initially was mostly non-embryogenic, and formation of embryogenic callus tissue was observed after two months in culture. Callus tissue cryopreserved by the other three combinations showed very little growth.

Figs. 51 and 52. Callus cultures of sugarcane plated in duplicates prior to freezing, after treatment with different cryoprotectants. Control tissue is plated at the center. Clockwise from arrow:
1. 5% DMSO.
2. 0.5 M sorbitol + 5% DMSO.
3. 0.5 M DMSO + 0.5 M glycerol + 1 M sucrose.
4. 10% PEG + 8% sucrose + 10% DMSO.
5. 5% glucose + 10% DMSO.

51. Callus tissue immediately after plating.

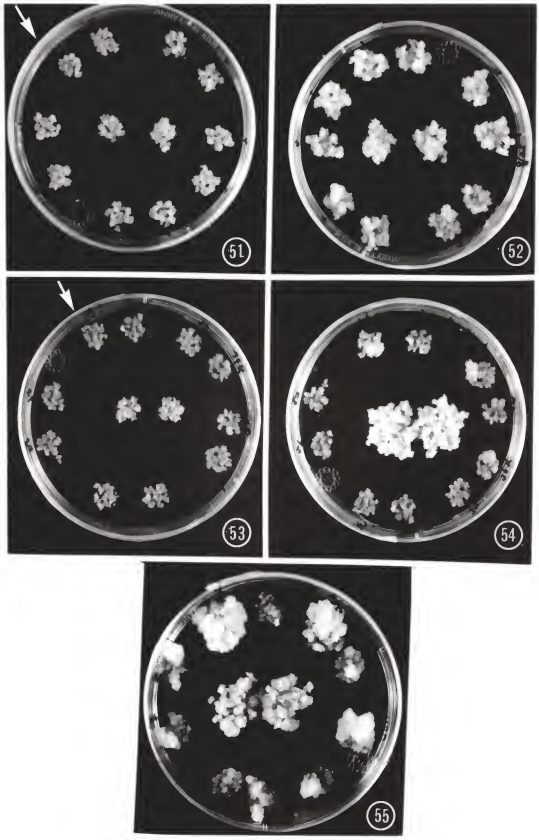
52. After one month.

Figs. 53-55. Control and cryopreserved callus tissue plated in the same order as Fig. 53. For each treatment, callus cultures were plated with and without washing. The tissue without washing is plated first in the clockwise direction for each treatment.

53. Callus tissue plated immediately after thawing.

54. After one month.

55. After two months.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52
53
54
55

Washing was deleterious in all cases and little or no growth took place when callus cultures were washed before plating (Fig. 54, 55). This was again confirmed from results obtained from the fresh weight assay (Table 13).

Callus tissue cryopreserved with 5% DMSO, cooled at a rate of 0.5°C/min, stored in liquid nitrogen, thawed rapidly and plated on semi-solid MS3C medium without removal of the cryoprotectants, exhibited very little growth compared to the control (Fig. 56). After three months in culture the growth rate of cryopreserved tissue was found to be comparable to that of the control (Fig. 57).

When transferred to regeneration media immediately upon thawing, or after a recovery period in MS3C medium, the callus always turned brown. No healthy tissue could be recovered after a two week period in this culture medium. Cryopreserving the calli by the method used for cryopreservation of cell suspension (transferring to liquid MS3C and then adding double strength cryoprotectant solution) gave similar results.

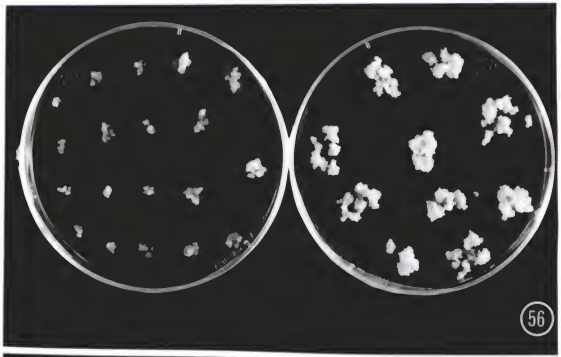
No difference in survival was observed among callus tissue of different sizes. Smaller pieces (1 mm), when plated singly did not produce any growth, but when three or four pieces were plated together growth was resumed.

Pregrowing the cell cultures in semi-solid MS3C supplemented with 5% DMSO, before treating with 5% DMSO and then freezing did not enhance survival. When thawed and plated the growth obtained was similar to growth obtained when calli were frozen in the presence of 5% DMSO without any pregrowth treatments.

Callus cultures frozen by direct immersion into liquid nitrogen, or frozen after removal of cryoprotectant by blotting dry did not survive freezing . When thawed and plated they became spongy and turned brown within a week. The embryogenic callus tissue recovered from calli cryopreserved with 5% DMSO started to grow after a lag period of 3-4 weeks and grew at rates comparable to the control. Plant regeneration, however, could not be achieved. When embryogenic

Fig. 56. Cryopreserved (L) and Control (R) tissue photographed after one month in culture. Cryopreserved tissue is plated immediately after thawing.

Fig. 57. Same as Fig. 58, but the cryopreserved tissue was used three months after thawing.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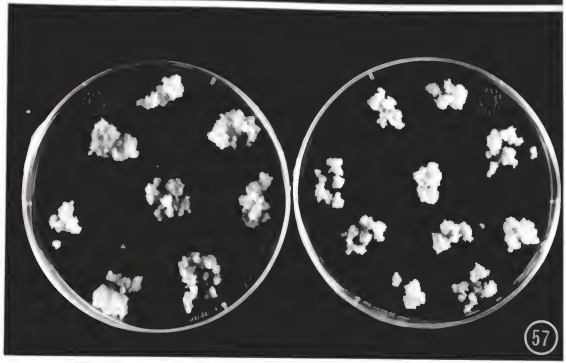
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callus was selected and transferred to regeneration medium, the callus tissue started to grow but turned brown in one week. No healthy tissue could be recovered from the cryopreserved tissue plated on regeneration medium after two weeks. When cryopreserved callus tissue was plated directly on the regeneration media, after thawing, the same results were obtained.

When callus was cryopreserved after a pregrowth period in 6% sorbitol, recovery was observed to be better than any of the previous procedures. When plated on MS3C medium, the cells resumed growth without a lag period and produced non-embryogenic tissue initially. Formation of embryogenic callus could be observed after two weeks, and the rate of growth of the callus was similar to that of the control (Fig. 58).

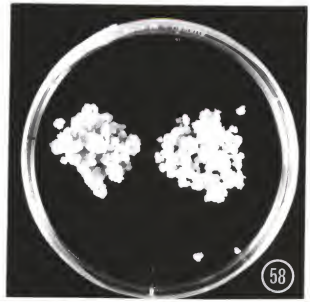
When cells were plated on the regeneration medium, removing the cryoprotectants by filter paper was invariably lethal. The cells turned brown, and no recovery was obtained in both MSB and MSSR media. When the callus was plated without removal of cryoprotectants, the tissue resumed growth immediately, and plants were recovered from both media. The growth and frequency of plant regeneration were comparable to the control tissue (Figs. 59, 60). Some browning was observed with both cryopreserved and control tissue when plated on MSB medium, but was completely absent in MSSR medium. It was better to incubate the callus tissue in light immediately after plating without a two week period in dark. When grown in the dark for two weeks before transfer to light the regeneration frequency declined.

Fig. 58-60. Cryopreserved (L) and control (R) tissue after one month in culture. Cryopreserved tissue was frozen after a pretreatment phase in MS3C + 6% mannitol, and plated without removal of the cryoprotectants.

58. On MS3C medium.

59. On MSB medium.

60. On MSSR medium.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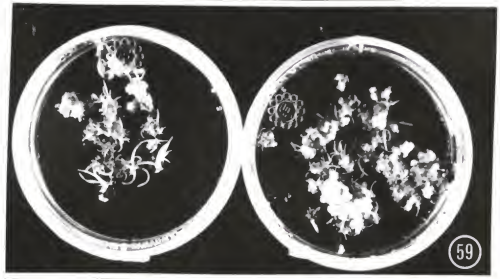
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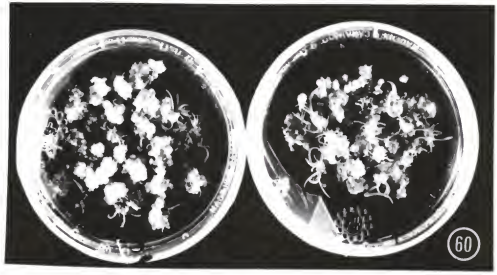
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Discussion

Supplementing the culture medium with osmotically active compounds has been shown to increase freeze tolerance, possibly by reducing the water content of the cell. A three to four day long pregrowth period in 6% mannitol or the closely related compound sorbitol is now a standard feature of cryopreservation procedures for cell suspensions (Withers 1985b), and has been used successfully in our laboratory for a number of grass species. When sugarcane cells were pregrown in mannitol, however, no recovery was obtained. Pregrowing the cells in 6% sorbitol improved the survival of sugarcane cell suspension. Improvement of viability has also been reported with cell suspension of *Nicotiana sylvestris* pregrown in a medium containing 6% sorbitol, when 6% mannitol was not appropriate (Maddox *et al.* 1983).

Survival of cell suspension based on TTC test could not be correlated with survival rates obtained from regrowth potential. Positive response with TTC could be due to the erratic behavior of the cells immediately after thawing. In addition, inorganic reactions are also known to mimic the production of formazan from TTC (Withers 1985b). This further supports the concept that survival should not be based on staining reaction alone. Over-estimation could be obtained when partially damaged cells give positive reaction but later die in culture, and under-estimation could result from cells in a cold shock or partially injured state, giving a negative reaction but later surviving in culture (Bajaj 1979a).

It is important to compare the results from the staining reaction with another viability test which involves growth parameters, to determine whether there is any positive correlation. Since no positive correlation was found in this study, percent regeneration was used as an indication of viability.

Using a mixture of cryoprotectants has been reported to be superior to using a single compound, by a reduction of the concentration of compounds possessing toxic effects towards the cell (Finkle and Ulrich 1978, 1979; Hauptmann and Widholm 1982; Withers 1982, 1985b; Bajaj 1983; Chen *et al.* 1984a,b; Finkle *et al.* 1985a; Kartha *et al.* 1988). Mixtures of 0.5 M glycerol, 0.5 M DMSO and 1 M sucrose (Withers 1985b) and 0.5 M sorbitol and 5% DMSO are commonly used in cryopreservation of cell cultures.

Majority of work on cryopreservation of callus cultures has been reported by Finkle and colleagues. They used a combination of 10% PEG, 8% glucose and 10% DMSO to decrease toxic effects which could result from using a single compound (Ulrich *et al.* 1979, 1984a,b,c; Tisserat *et al.* 1981). Callus cultures of date palm (Finkle *et al.* 1979; Tisserat *et al.* 1981), rice (Finkle and Ulrich 1982; Finkle *et al.* 1983), sugarcane (Ulrich *et al.* 1979, 1984a) and American elm (Ulrich *et al.* 1984c) could be cryopreserved successfully using this combination of cryoprotectants, and plants were recovered from the cryopreserved tissue of date palm and American elm callus cultures.

Using a single cryoprotectant (5% DMSO) was superior to using a mixture of cryoprotectants for cryopreservation of sugarcane callus in our work. Low survival was obtained when a combination of PEG, glucose and DMSO was used. Resumption of growth and regeneration of plants were obtained from callus cultures of wheat cryopreserved with 0.5 M sorbitol and 5% DMSO (Chen *et al.* 1985). This combination gave satisfactory results but growth was better when 5% DMSO was used. Differentiation and metabolic potential of green *Lavandula vera* callus culture could be retained by cryopreserving them in the presence of 5% DMSO and 10% glucose (Watanabe *et al.* 1983). When used for cryopreservation of sugarcane callus cultures, survival was found to be poor.

Slow freezing is generally employed for cryopreservation of cell suspension (Withers 1985b) and callus cultures. Callus cultures are generally frozen at a slow rate to sub-zero temperatures before transfer to liquid nitrogen (Ulrich *et al.* 1979, 1984b,c; Tisserat *et al.* 1981; Finkle and Ulrich 1982; Chen *et al.* 1985). Chen *et al.* (1985) used a rate of 0.5°C/min for cryopreservation of callus cultures of wheat. A rate of 1°C/min was used for cryopreservation experiments by Finkle and colleagues (Tisserat *et al.* 1981; Finkle and Ulrich 1982; Ulrich *et al.* 1984b,c), and direct immersion was found to be lethal (Ulrich *et al.* 1979).

A rate of 0.5°C/min was used for freezing of callus and cell suspension in the present study. It has been found to be optimal for several species (Chen *et al.* 1985; Kartha 1985a), including cell suspension cultures of *Panicum maximum*, *Pennisetum americanum*, and *Pennisetum purpureum* (See chapter 4). When callus was immersed directly in liquid nitrogen and plated after thawing it became spongy and turned brown after a few days. No recovery was obtained.

Rapid thawing was employed for both callus cultures and cell suspension cultures to avoid damage to cells by recrystallization of ice. Post-thaw treatment is a very important step for recovery of cryopreserved cells. In previous work done with cryopreservation of callus, the tissue was washed before plating with hormone free liquid medium (Chen *et al.* 1985), or the cryoprotectants were diluted gradually with a wash solution composed of major MS mineral salts and sucrose (Ulrich *et al.* 1979, 1984c; Tisserat *et al.* 1981; Finkle and Ulrich 1982). Finkle and Ulrich (1982) reported that survival of callus tissue after cryopreservation was better when washed at 22°C, rather than at 0°C. With sugarcane callus and suspension cultures, post-thaw washing was found to be invariably damaging, but washing at 0°C was better than washing at room temperature (Table 12). Blotting dry the excess cryoprotectants from the callus, and plating the cell suspension culture on filter paper for gradual removal of cryoprotectants was found to be superior to post-thaw

washing. But with callus cultures, the filter paper technique was inhibitory for recovery. It was essential to plate the callus without removal of the cryoprotectant solution. Water, ions, sugar and amino acids could be lost during freezing, and washing would cause removal of these compounds which may be required for the recovery of the cells (Withers and King 1979b). Washing the cells could also cause deplasmolysis injury, resulting from the loss of membrane material during freezing (Wiest and Steponkus 1978; Withers and King 1979b; Withers 1985b).

Pregrowth treatment is generally not employed in cryopreservation of callus tissues because of their growth habit which impedes any pregrowth treatments (Withers 1986). Plants were obtained from sugarcane callus cultures frozen to -23°C (Ulrich *et al.* 1979), but when frozen to liquid nitrogen temperatures the plants recovered were invariably albinos which grew to only a few cm in height (Ulrich *et al.* 1984a). When callus tissue was frozen without a pregrowth period, although survival was obtained, the cells grew after an extended lag phase and no plants could be recovered.

Freeze tolerance is increased by growing the cells in a medium with high osmoticum, by reduction in cell size, vacuolar volume and consequently water content (Withers 1985b). Pregrowing the callus tissue in a liquid medium supplemented with 6% sorbitol for two days improved the survival considerably. There was no lag period, and the tissue resumed growth after 2-3 days and grew at rates equivalent to that of the control. Healthy plants of normal morphology were also recovered from this cryopreserved tissue.

Sugarcane suspension and callus culture could be frozen to liquid nitrogen temperatures without any loss in their morphogenetic potential. Although a number of studies have reported cryopreservation of sugarcane cells, regeneration has not been achieved. By introducing a pregrowth period in the growth medium

supplemented with sorbitol, using a slow cooling rate, and avoiding post-thaw washing considerable improvement was achieved in the viabilities.

CHAPTER 6

PLANT REGENERATION FROM CRYOPRESERVED IMMATURE EMBRYOS OF WHEAT (TRITICUM AESTIVUM L.)

Introduction

Cryopreservation of embryos can be useful in germplasm preservation. Various types of embryos such as zygotic, somatic, nucellar and pollen embryos have been successfully cryopreserved. Cryopreservation of zygotic embryos may be an alternative for plantation crops and a number of fruit and timber trees, which have recalcitrant seeds that cannot be preserved under ordinary conditions (Bajaj 1985).

Materials and Methods

Plant Material

Immature seeds of a commercial wheat cultivar (Triticum aestivum L.) were collected from the field (Central Plain Experimental Station, Tifton, GA) 10-12 days after pollination. They were surface sterilized in 70% alcohol for 30 seconds, followed by immersion in 20% chlorox for 15 minutes, then rinsed with four changes of sterile distilled water.

Immature embryos, 0.5-1.5 mm in size, were excised under a dissecting microscope, and cultured on basal MS (Murashige and Skoog 1962) medium

containing, 2 mg/l 2,4-D, 3% sucrose, 100 mg/l caesin hydrolysate, and 500 mg/l glutamine (pH adjusted to 5.8 before autoclaving) gelled with 0.2% gelrite. The embryos were placed with the embryo axis in contact with the surface of the medium, and incubated in total darkness at 28°C.

Age of the Explant

The immature embryos were cryopreserved either immediately after isolation, or one to three days after culture.

Pretreatment

Three different pretreatments were tried.

a. The excised embryos were cultured from five hours to three days on semi-solid culture medium containing 5% DMSO.
b. The embryos were cultured on media with increasing concentrations of sucrose (3%, 10%, 15%, and 20%, w/v) for one to three days.
c. The entire inflorescence was pretreated in the refrigerator (4°C) for five days before removal of the embryos.

Cryoprotectants

The following cryoprotectants were used, either singly or in combination.

1. 5% DMSO
2. 0.5 M sorbitol + 5% DMSO
3. 0.5 M glycerol + 0.5 M DMSO + 1.0 M Sucrose
4. 10% DMSO + 4% sucrose

Before treatment with the cryoprotectants, the embryos were transferred from the semi-solid culture medium into chilled liquid culture medium of the same composition, in an Erlenmeyer flask. Equal volume of the cryoprotectant solution

was prepared at double strength in water, filter sterilized, chilled on ice, and added gradually over a period of one hour, with stirring, to the chilled culture medium containing the embryos. The Erlenmeyer flask containing the embryos in the cryoprotectant solution was then placed on a rotary shaker at 4°C for an hour to facilitate the uptake of cryoprotectants.

Freezing

The embryos were frozen by the following methods:

1. Slow freezing
2. Rapid freezing
3. Dry freezing

Slow freezing. One ml of the solution containing the embryos was transferred to 1.2 ml presterilized, polypropylene screw-cap ampoules. They were cooled at a rate of 0.5°C/min in a Cryomed Model 1010 Micro Computer Programmable Freezer Unit. The temperature was monitored by placing a thermocouple in one of the ampoules. Slow cooling was terminated at -40°C and the ampoules were transferred to liquid nitrogen after a holding period of 40 minutes.

Rapid freezing. The embryos were cooled rapidly by plunging the ampoules directly into liquid nitrogen from 0°C.

Dry freezing. The cryoprotectant treated embryos were blotted dry on sterile filter paper to remove surface moisture, enclosed in aluminum foil envelopes and plunged directly into liquid nitrogen.

Thawing

After storage in liquid nitrogen for two weeks, the ampoules were thawed rapidly by transferring to water at 40°C for 45-60 seconds with constant agitation. The cryoprotectants were removed from the embryos either by washing with liquid medium or by blotting dry with sterile filter paper, and then plated with the embryo axis down on the same medium used for unfrozen controls. They were then incubated at 28°C in total darkness and scored for formation of callus.

Regeneration

Embryogenic calli formed from the immature embryos were isolated and plated on regeneration medium (MS basal + 2% sucrose + 1 mg/l IAA (indole acetic acid) + 1 mg/l Zeatin) and maintained at 28°C with a 16 hr photoperiod. Regenerated shoots were transferred to rooting medium (MS basal + 2% sucrose + 0.5 mg/l IAA + 0.04 mg/l Zeatin). After the formation of an adequate root system, the plantlets were transferred to soil in conetainers and left for a month in a moisture chamber, after which they were transferred to pots and grown in the greenhouse.

Results

When immature embryos were dissected and placed on semi-solid medium the scutellum started to swell in three to four days. Formation of callus on the enlarged scutellum could be observed after 7-10 days. In approximately three weeks two types of calli - embryogenic and non-embryogenic - could be distinguished. The embryogenic callus was off-white, compact, nodular and could be easily distinguished from the white and less compact non-embryogenic callus.

Age of the Explant

No callus was formed even after two months when embryos were cryopreserved immediately after excision. The embryos did not become brown, but also showed no signs of growth. Only 16% of the embryos survived when they were cryopreserved after one day of culture. Best results (69% survival) were obtained when the embryos were cryopreserved after three days in culture (Table 14).

Pretreatment

Pretreating the embryos by growing them on semi-solid medium with 5% DMSO did not enhance survival. Only non-embryogenic callus was formed following pretreatment with DMSO for 5-24 hours. The response worsened with longer periods of exposure to DMSO (Table 15). The embryos pretreated with DMSO did not produce any callus even without freezing.

Embryos freeze hardened by culture in a medium with high osmoticum (10-20% sucrose) produced no embryogenic callus after cryopreservation (Table 16). The calli formed on these media were more compact, yellowish and showed reduced growth compared to the controls. Even unfrozen embryos treated with cryoprotectants, had a reduced potential to produce embryogenic callus.

During cold pretreatment many of the embryos were found to develop to a stage more mature than what is normally used for culture. Even when embryos at the right stage were selected and used for cryopreservation, they did not show any improvement on survival compared to controls.

Cryoprotectants

Embryogenic callus was recovered from cryopreserved embryos when 5% DMSO or 10% DMSO + 8% sucrose were used as cryoprotectants. A higher

Table 14: Percent survival of embryos (based on formation of callus) cryopreserved at different time intervals after excision (averages of two experiments, 30 embryos per treatment).

Time after excision	Formation of callus (%)*	Embryogenic callus (%)*	Non-embryogenic callus (%)*
One hour	00±0.00	00±0.00	00±0.00
One day	16±1.30	03±0.70	13±4.19
Two days	53±5.02	13±2.64	40±3.32
Three days	69±5.14	36±4.17	33±3.33

* Numbers represent the values of the mean ± SEM

Table 15: Percent survival of embryos (based on formation of callus) pretreated with 5% DMSO for different lengths of time. The embryos pretreated for 5 hours were cultured for a day in regular medium before transfer to DMSO (averages of two experiments, 30 embryos per treatment).

Time in 5% DMSO	Formation of callus (%)*	Embryogenic callus (%)*	Non-embryogenic callus (%)*
5 hours	26±4.41	00±0.00	26±4.41
One day	13±2.08	00±0.00	13±2.08
Two days	00±0.00	00±0.00	00±0.00
Three days	00±0.00	00±0.00	00±0.00

* Numbers represent the values of the mean ± SEM

Table 16: Survival of embryos (based on callus formation) pregrown on media with high osmoticum for 1-3 days (averages of two experiments, 30 embryos per treatment).

Time (days)	Percent sucrose	Formation of callus (%)*	Embryogenic callus (%)*	Non-embryogenic callus (%)*
1	3	46±4.09	06±4.69	40±8.75
	10	13±4.09	00±0.00	13±4.09
	15	07±5.99	00±0.00	07±5.99
	20	13±3.04	00±0.00	13±3.04
2	3	53±4.3	20±2.61	33±5.63
	10	13±7.11	00±0.00	13±7.11
	15	00±0.00	00±0.00	00±0.00
	20	00±0.00	00±0.00	00±0.00
3	3	40±5.61	07±4.71	33±1.79
	10	33±4.08	00±0.00	33±4.08
	15	20±6.13	00±0.00	20±6.13
	20	07±3.76	00±0.00	07±3.76

* Numbers represent the values of the mean ± SEM

incidence of embryogenic callus was observed when 5% DMSO was used, than when 10% DMSO + 8% sucrose were used as cryoprotectants (Table 17). When 0.5 M sorbitol + 5% DMSO were used, there was no response. The embryos remained quiescent for long periods of time. Mostly non-embryogenic callus was produced when 0.5 M glycerol + 0.5 M DMSO + 1.0 M sucrose were used.

Freezing

Embryogenic callus formation was observed only when embryos were frozen at slow rates to sub-zero temperatures and then plunged into liquid nitrogen. When cooled rapidly by plunging directly into liquid nitrogen, most of the embryos formed a single type of callus which was loose and watery, and could not be subcultured. This type of callus was not observed with the unfrozen control embryos. No swelling was observed in the scutellum and the callus was formed from the coleoptile.

When embryos were frozen in foil envelopes, there was no recovery at all. In addition, contamination was a consistent problem.

Thawing and Regrowth

Greater recovery of viable embryos was obtained when cryoprotectants were removed before plating the explants by blotting dry with sterile filter paper, than by washing with liquid culture medium. There was a lag period of 7-10 days before any signs of growth could be observed. Callus formation and growth rate were similar to those of the control.

Plant Regeneration

The off white, compact and nodular embryogenic callus (Figs. 61, 62) was separated from the non-embryogenic callus after a month and transferred to maintenance as well as regeneration media. Shoot formation could be observed in

regeneration medium after 10-14 days (Fig. 63). Two to six plantlets were recovered from each of the cryopreserved and control embryos. The shoots that were at least 0.5-1 cm high after one month in culture (Fig. 64) were transferred to rooting medium in Petri dishes, and the rest of the calli along with the developing shoots were transferred to fresh regeneration medium for further development of the shoots before transfer to rooting medium. After one month the shoots in rooting medium (Fig. 65) were again transferred to the same medium in Magenta boxes to facilitate further root growth (Fig. 66).

After the formation of an adequate root system (4-6 weeks), the plantlets were transferred to soil in Conetainers (Ray Leach Conetainer Nursery), and grown in a growth chamber under high humidity. After a month, the plants were ready to be transferred to soil in both clay and plastic pots, and transferred to the greenhouse. Transferring the plantlets to soil in pots directly from the Magenta boxes, without a period of growth in Conetainers, caused severe reduction in growth.

The plants from cryopreserved embryos were morphologically similar to the control plants (Fig. 67). Cryopreserved and control plants in plastic pots grew vigorously but did not set seeds (Figs. 67, 68). Whereas the plants in clay pots, both control and cryopreserved, grew into plants that were not very vigorous. But seed setting occurred after two months (Fig. 69).

Table 17: Effect of different cryoprotectants on survival of cryopreserved wheat embryos (averages of two experiments, 30 embryos per treatment).

Cryoprotectants	Formation of callus (%)*	Embryogenic callus (%)*	Non-embryogenic callus (%)*
5% DMSO	66±4.24	33±3.45	33±5.46
0.5 M sorbitol + 5% DMSO	00±0.00	00±0.00	00±0.00
0.5 M glycerol + 0.5 M DMSO + 1 M sucrose	33±5.40	03±2.35	30±3.32
10 % DMSO 4% sucrose	56±5.65	16±3.89	40±6.58
Control	86±4.31	82±2.97	04±2.04

* Numbers represent the values of the mean ± SEM

Fig. 61. Cryopreserved embryos showing formation of callus on maintenance medium.

Fig. 62. Formation of embryogenic and non-embryogenic callus from a cryopreserved immature embryo.

Fig. 63. Formation of shoots from a cryopreserved immature embryo on regeneration medium.

Fig. 64. Immature embryos on regeneration medium showing formation of shoots and roots.

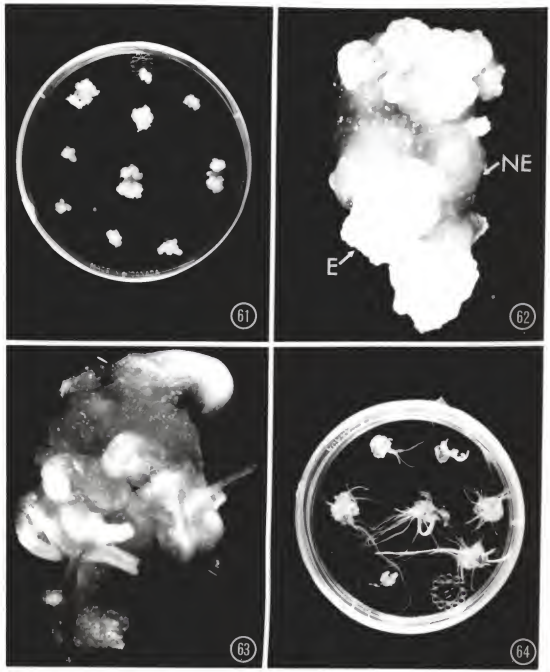
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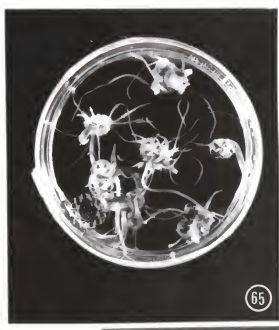
Fig. 65. Immature embryos on rooting medium in Petri dishes.

Fig. 66. A plantlet from cryopreserved embryo on rooting medium in a Magenta box.

Fig. 67. Plants from cryopreserved (L) and control (R) embryos grown to maturity in the green house (2 months old).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66


67

Fig. 68. A four month old wheat plant from a cryopreserved embryo, grown to maturity in the green house in a clay pot. The plant did not seeds.

Fig. 69. Cryopreserved and control plants grown to maturity in plastic pots showing seed set.


68
69

Discussion

Wheat embryos produce embryogenic callus which is ready to be transferred to fresh medium after a month, but after 3-4 subcultures, the embryogenic cultures become more and more friable and loose their embryogenic capacity (Redway *et al.* 1990). Continuous supply of embryogenic calli is often needed to initiate embryogenic cell suspensions for the isolation of protoplasts and for genetic transformation (Redway *et al.* 1990; Vasil *et al.* 1990). For this reason, it would be very useful if the immature embryos could be cryopreserved to provide a year round supply.

In this study it is shown that immature embryos can be frozen to liquid nitrogen temperatures and still retain their capacity to form embryogenic callus which is capable of regenerating normal plants.

The embryos could not be cryopreserved immediately after excision. In addition, contamination was a constant threat as the presence of even a single contaminated embryo would spoil the whole batch of embryos when they were pooled together. Highest survival was obtained when the embryos were frozen after three days in culture. At this time the contaminated embryos could be detected and discarded. Scutellum started to swell by the third to fourth day, but embryos with enlarged scutella could not be cryopreserved. After three days in culture it was possible to select the embryos that were not contaminated and did not show any signs of an enlarged scutellum. After four days in culture most of the embryos showed swelling. Therefore, embryos are best used three days after excision.

Various pretreatments have been used for embryo cryopreservation. Freeze hardening the embryos by either leaving them in the kernels or by culturing the excised embryos in high osmotica was successfully used for the cryopreservation of maize embryos, when common cryoprotective treatment failed (Delvallee *et al.*

1989). But when freeze hardening was used for the cryopreservation of wheat embryos, the embryos that survived did not produce embryogenic callus. Callus growth was retarded in high osmotica and the embryogenic callus formed was morphologically different from that of the control.

Various cryoprotectants have been used for the cryopreservation of embryos. When zygotic embryos of wheat were cryopreserved with 10% DMSO and 4% sucrose, 41-48% survival based on the ability of the frozen embryos to increase in size, proliferate to form callus and develop roots, shoots, and plantlets was obtained (Bajaj 1984). The embryos were cryopreserved immediately after excision by rapid freezing. The immature embryos used in the present study could not be cryopreserved successfully by this method. Irrespective of the cryoprotectant, cooling rate, etc., there was no recovery when the embryos were frozen immediately after excision. In addition, when rapid rates of cooling (direct immersion) were used, irrespective of the cryoprotectants used, the callus recovered was loose and watery and could not be subcultured. When the same combination of cryoprotectants (10% DMSO + 4% sucrose) were used with slow rate of cooling three days after excision of the embryos 56% of the embryos survived of which only 16% were capable of producing embryogenic callus. In contrast, when the embryos were frozen slowly by using 5% DMSO as the sole cryoprotectant, 66% of the embryos survived with 33% capable of forming embryogenic callus. Plants could be regenerated from the embryogenic calli produced from these cryopreserved embryos.

Combinations of cryoprotectants are generally better than single compounds (Finkle and Ulrich 1979; Kartha *et al.* 1988). No embryos survived when 0.5 M sorbitol + 5% DMSO were used as cryoprotectants, although it was proven to be the best combination for the cryopreservation of a number of cell suspensions (See chapter 2). A combination 0.5 M glycerol + 0.5 M DMSO + 1 M sucrose,

commonly used in cryopreservation experiments (Withers 1984b, 1985b), was also not suitable for cryopreservation of wheat immature embryos.

Best survival was obtained when DMSO was used singly at a concentration of 5%. DMSO is considered to be an efficient cryoprotectant because of its low molecular weight, good miscibility with water, easy removal by washing and rapid permeation into cells (Bajaj 1985). Concentrations of 5-10% have been proven to be optimum in a number of studies (Latta 1971; Nag and Street 1973, 1975a; Dougall and Wetherell 1974), and have been commonly employed for cryopreservation of embryos. It has also been used successfully for the cryopreservation of somatic embryos and clonal plantlets of carrot (*Daucus carota* L.) at concentrations of 2.5 to 20% (v/v) (Withers 1979), and for pollen embryos of *Atropa* and *Nicotiana* at concentrations of 5-7% (Bajaj 1977).

Cells are damaged by plasmolysis when high concentrations of DMSO are used. DMSO (5-10%) added to carrot cell suspensions did not cause any adverse effects up to one hour at 3°C, but at 23°C the rate of survival without freezing dropped continuously with time (Bajaj and Reinert 1977). To prevent excessive plasmolysis of the cells it is best to add the cryoprotectants gradually at chilling temperatures (Withers and Street 1977a).

Although used for cryopreservation of embryos of a number of species, rapid cooling was not suitable for wheat embryos. Similar survival percentages were obtained with rapid and slow cooling, when zygotic embryos of cassava were cryopreserved (Marin *et al.* 1990). In the present study embryos cooled rapidly failed to produce any embryogenic callus. Freezing should be such that intracellular ice crystal formation does not take place and all freezable water is removed from the cell during cooling (Mazur 1970). With rapid cooling ice crystals that are too small to cause any lethal damage are formed during freezing, but these undergo disruptive recrystallization during thawing, causing damage to cells (Withers 1980).

Slow cooling is preferable because of the occurrence of reduction in cell volume which leads to reduced intracellular ice crystal formation, preventing cell damage. A net rate of 0.5-1°C/min is generally suitable for cryopreservation of cell suspension cultures (Withers 1987). Limited availability of material prevented extensive experimenting with cooling rates in this study. A rate of 0.5°C/min gave satisfactory results.

Somatic embryos of carrot (*Daucus carota* L.) were successfully preserved by removing the superficial moisture and enclosing them in foil envelopes before freezing (Withers 1979). Wheat embryos cryopreserved by this method failed to survive, in addition to the problems of contamination.

In plant and animal tissues at -30°C all freezable water is withdrawn from the cells by extracellular freezing. When they survive this temperature the percent recovery is normally not much affected by ultra-low temperatures (Sakai 1960). Only 5% survival was recorded in suspension cultures of sycamore frozen to -23°C before immersion in liquid nitrogen, whereas 20-30% of the cells survived when the holding temperature was decreased to -40 or -50°C. Clonal plantlets of carrot (*Daucus carota* L.) cryopreserved by slow cooling, did not survive when the terminal freezing temperature was -30°C, but -40° was found to be suitable for cryopreservation (Withers 1979).

Slow cooling can be terminated at a suitable sub-zero temperature and the material can be transferred to liquid nitrogen immediately or after a holding period, which permits cellular protective dehydration (Withers 1978a, 1984b). In the present study slow cooling was terminated at -40°C and the ampoules were transferred to liquid nitrogen after a holding period of 40 minutes.

The embryos were stored in liquid nitrogen at least for two weeks before thawing. Thawing can be done either rapidly in a water bath at 40°C or slowly at room temperature. Rapid thawing is recommended (Withers 1986) because

damage to cells by recrystallization of the ice crystals present in the cell is prevented (Withers 1980).

Washing of freshly thawed cells is mostly deleterious and is never beneficial (Withers 1984a). When wheat embryos were washed with the culture medium the percentage survival declined. Staining reactions alone are not a reliable means of viability. Survival of embryos should be based on resumption of growth and the ability to form callus. Thus, a viability of 66-69% was obtained when embryos were cryopreserved three days after excision using 5% DMSO as cryoprotectant and frozen by slow cooling. Thirty three to thirty six percent of these embryos retained the ability to produce embryogenic callus. The embryogenic calli formed were morphologically identical to those of the controls, and produced shoots at similar frequencies. The plants recovered were grown to maturity in the greenhouse.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Methods were developed and optimized for the successful cryopreservation of cell suspensions of *Panicum maximum* and *Pennisetum americanum*. Cell viability was improved by pregrowing the cells in a medium supplemented with 6% (w/v) mannitol and cryoprotecting with 0.5 M sorbitol and 5% DMSO. The use of an uptake period, after cryoprotectant addition but prior to freezing, failed to improve survival. A cooling rate of 0.5°C/min produced optimum results. Viability decreased with increasing cooling rates, and rapid freezing by direct immersion in liquid nitrogen was always lethal. A transfer temperature of -40°C gave the optimum results and holding the cells at sub-zero temperature before transfer to liquid nitrogen caused reduction in viability.

This optimized procedure could be used for the successful cryopreservation of a number a Gramineous species, without the requirement for any preliminary experiments. It also served as a selection process by which small, highly cytoplasmic embryogenic cells were preferentially selected over large, highly vacuolated non-embryogenic cells. The cell suspension recovered from the cryopreserved cells, hence were more suitable for protoplast isolation.

For cell suspension of sugarcane, mannitol could not be used as an osmoticum to improve survival, but sorbitol was found to be appropriate for this purpose. Removal of cryoprotectants before plating was essential for recovery. Washing was deleterious, but gradual removal of the cryoprotectants by a filter paper technique ensured success. The regeneration frequency of the cryopreserved

cells was 92% of the control. Plants recovered from the cryopreserved cells were grown to maturity in the greenhouse and found to be similar to the control.

For callus cultures the use of a single cryoprotectant (5% DMSO) yielded better results than a mixture of cryoprotectants. They resumed growth after a lag period of one month. Embryogenic calli produced from these tissues failed to regenerate plants. By including a pregrowth period in liquid culture medium supplemented with 6% sorbitol, considerable improvement was obtained in viability. The cryopreserved tissue resumed growth without a lag period and regenerated plants at frequencies equal to the control. It was critical to culture the callus tissue without the removal of the cryoprotectants.

TTC reduction assay, used extensively for determining the viability of cell suspensions, was not reliable for sugarcane suspension. With *Panicum maximum* and *Pennisetum americanum* although positive correlation was observed with regrowth potential, under-estimation was apparent. Hence, this method should not be used alone for determination of viability, and the results should be verified by using a growth parameter such as regrowth potential.

Optimal results were obtained when immature embryos were cryopreserved after three days in culture. Freezing them immediately after isolation was invariably lethal. Again, 5% DMSO was superior to using a mixture of cryoprotectants. Freeze hardening the embryos by a cold pretreatment in a high osmotic medium failed to enhance survival. Freezing by direct immersion or dry freezing in aluminum foil envelopes was lethal. Plants recovered from the cryopreserved embryos were fertile and set seeds when grown to maturity in the greenhouse.

A cell suspension of *Panicum maximum* was used for ultrastructural and morphometric studies. Cells pregrown in high osmoticum had a size reduction of 11%. The vacuolar volume was reduced to only 23% of the cell volume, as compared to 56% in the control cells. The plasma membrane formed invaginations

to accommodate the reduction in cell size. Dilation of cell organelles was also observed at different stages of the cryopreservation procedure. In cells frozen after treatment with the cryoprotectants most membranous structures were found intact, although some damage was apparent. These cells when fixed after two days in culture showed normal morphology, indicating their potential to repair these damages. On semi-solid medium they resumed growth and grew at rates comparable to that of the control. Cells frozen without any cryoprotection or frozen rapidly after cryoprotection were lethally damaged. These cells lacked most membranous structures, and did not resume growth.

The present work extends our knowledge of the factors involved in cryopreservation of cell suspensions, callus cultures and explants in the Gramineae. The morphogenetic potential of the tissue is not lost after freezing to liquid nitrogen temperatures. Pregrowth treatment in high osmoticum and treatment with cryoprotective compounds are essential for recovery. The extent of cell damage is considerably reduced by pregrowth, cryoprotectant treatment and by slow freezing, and cells are able to repair these damages in a short period of time.

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